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AF/1635

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19

October 8, 2003

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Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Mail Stop Appeal Brief-Patents
Art Unit 1635

Re: U.S. Utility Patent Application
Appl. No. 09/964,678; Filed: September 28, 2001
For: **Transgenic Animals and Cell Lines for Screening Drugs Effective for the Treatment or Prevention of Alzheimer's Disease**
Inventors: de la Monte *et al.*
Our Ref: 0609.4370002/RWE/FRC

Sir:

Transmitted herewith for appropriate action are the following documents:

1. Fee Transmittal Form (PTO/SB/17);

2. Brief on Appeal Under 37 C.F.R. § 1.192 (in triplicate) along with Exhibits 1-18;

3. PTO-2038 Credit Card Payment Form for **$165.00** to cover the Brief filing fee; and

4. Return postcard.

It is respectfully requested that the attached postcard be stamped with the date of filing of these documents, and that it be returned to our courier. In the event that extensions of time are necessary to prevent abandonment of this patent application, then such extensions of time are hereby petitioned.

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Frank R. Cottingham
Attorney for Appellants
Registration No. 50,437

FRC/pcd
Enclosures

::ODMA\MHODMA\SKGF_DC1;186278;1

PTO/SB/17 (08-03)
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FEE TRANSMITTAL for FY 2003

Effective 01/01/2003. Patent fees are subject to annual revision.

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($)165.00

Complete if Known	
Application Number	09/964,678
Filing Date	Sept mber 28, 2001
First Named Inventor	Suzann de la Monte
Examiner Name	Whit man, B.
Art Unit	1635
Attorney Docket No.	0609.4370002/RWE/FRC

OIPE
OCT 0 8 2003
PATENT & TRADEMARK OFFICE

METHOD OF PAYMENT (check all that apply)

☐ Check ☒ Credit card ☐ Money Order ☒ Other** ☐ None

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☐ Deposit Account
Deposit Account Number 19-0036
Deposit Account Name: Sterne, Kessler, Goldstein & Fox P.L.L.C.

The Commissioner is authorized to: *(check all that apply)*

☒ Charge fee(s) indicated below ☒ Credit any over payments

☒ Charge any additional fee(s) during the pendency of this application

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1105	160	2005	80	Provisional filing fee	

SUBTOTAL (1) ($) _____

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra		Fee from below		Fee Paid
Total Claims _____ - 20** =	_____	X	_____	=	_____
Indep. Claims _____ - 3** =	_____	X	_____	=	_____
Multiple Dependent	_____			=	_____

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	**Reissue independent claims over original patent
1205	18	2205	9	**Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)_____

***or number previously paid, if greater; For Reissue, see above*

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1502	50	2052	25	Surcharge-late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	**330**	**2402**	**165**	**Filing a brief in support of an appeal**	**165.00**
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

* Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($)165.00

SUBMITTED BY

Name (Print/Type)	Frank R. Cottingham	Registration No. (Attorney/Agent)	50,437	Complete *(if applicable)* Telephone	202-371-2600
Signature	Frank R. Cottingham			Date	OCT. 08, 2003

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

This collection of information is required by 37 CFR 1.17 and 1.27. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

de la MONTE *et al.*

Appl. No. 09/964,678

Filed: September 28, 2001

For: **Transgenic Animals and Cell Lines for Screening Drugs Effective for the Treatment or Prevention of Alzheimer's Disease**

Confirmation No.: 3649

Art Unit: 1635

Examiner: Whiteman, B.

Atty. Docket: 0609.4370002/RWE/FRC

Brief on Appeal Under 37 C.F.R. § 1.192

Mail Stop Appeal Brief - Patents

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

A Notice of Appeal from the final rejection of claims 7-9, 14-16 and 35-40 was filed on August 8, 2003. Appellants hereby file this Appeal Brief in triplicate, together with the required brief filing fee.

I. Real Parties in Interest (37 C.F.R. § 1.192(c)(1))

The real parties in interest in this appeal are The General Hospital Corporation and Nymox Corporation.

II. Related Appeals and Interferences (37 C.F.R. § 1.192(c)(2))

Appellants' undersigned representative is not aware of any appeals or interferences related to the captioned application.

III. Status of Claims (37 C.F.R. § 1.192(c)(3))

The captioned application was filed on September 28, 2001 as a divisional of U.S. Patent Application No. 09/380,203, which is currently pending. As originally filed, this application contained a total of 34 claims.

In a Preliminary Amendment filed on September 28, 2001, claims 1-6, 10-13 and 17-34 were cancelled.

In an Amendment filed on August 8, 2002, claims 7, 8 and 15 were amended, and claims 35-36 were added.

In an Amendment filed on January 22, 2003, claims 7, 14 and 36 were amended, and claims 37-40 were added.

Claims 7-9, 14-16 and 35-40 are pending in this application and are now on appeal. A copy of the claims on appeal can be found in the attached Appendix.

IV. Status of Amendments (37 C.F.R. § 1.192(c)(4))

All amendments have been entered. No amendments have been filed subsequent to the issuance of the final Office Action dated April 8, 2003 (Paper No. 16).

V. Summary of Invention (37 C.F.R. § 1.192(c)(5))

Alzheimer's disease ("AD") is a neurodegenerative disease characterized by prominent atrophy of corticolimbic structures with neuronal loss, neurofibrillary tangle formation, aberrant proliferation of neurites, senile plaques and βA4-amyloid deposition in the brain. *See* Specification at page 2, lines 1-4. In previous studies, the inventors demonstrated that polyclonal antisera prepared against a pancreatic protein had increased immunoreactivity in the brains of AD patients. *See* Specification at page 5, lines 3-9. Using the polyclonal antisera, a cDNA clone corresponding to the antigen recognized by the antisera was isolated from an AD brain expression library. *See* Specification at page 5, lines 9-11. The polypeptide encoded by the isolated cDNA was designated "AD7c-NTP" (NTP = neuronal thread protein). *See* Specification at page 17, lines 17-18. AD7c-NTP is expressed in neurons and is over-expressed in brains of AD patients. *See id.* Abnormal expression of AD7c-NTP is a phenotype associated with AD. *See* Specification at page 18, lines 7-9.

The nucleotide sequence of the AD7c-NTP gene is set forth in the application as SEQ ID NO:1. The amino acid sequence of AD7c-NTP is set forth in the application as SEQ ID NO:2. *See* Specification at page 7, lines 16-17, and Figs. 1A-1C.

Claims 7-9, 35, 36, 39 and 40 are directed to transgenic non-human animals, all of whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, wherein the DNA molecule is over-expressed in one or more cells of the transgenic animals, and wherein the DNA molecule codes for a protein that has an activity of AD7c-NTP when over-expressed in neuronal cells. Claim 8 specifies that the DNA molecule contained in each germ and somatic cell has SEQ

ID NO:1. Claim 9 specifies that the protein coded for by the DNA molecule is overexpressed in the brain of the animal. Support for claims 7-9 can be found throughout the specification, for example, at page 18, lines 15-18, at page 20, lines 1-3, and in claims 7-9 as originally filed. Claim 36 specifies that the activity of AD7c-NTP possessed by the DNA molecule when over-expressed in neuronal cells is selected from the group consisting of neuritic sprouting, nerve cell death, nerve cell degeneration, neurofibrillary tangles and irregular swollen neurites. Support for claim 36 can be found throughout the specification, for example, at page 22, lines 1-12. Claim 35 specifies that the transgenic non-human animal is selected from the group consisting of non-human primate, mouse, sheep, pig, cattle, goat, guinea pig and rat. Claim 39 specifies that the transgenic animal is a vertebrate. Claim 40 specifies that the transgenic animal is a mammal. Support for claims 35, 39 and 40 can be found throughout the specification, for example, at page 13, lines 7-12.

Claims 14-16 are directed to *in vitro* methods for screening a candidate drug that is potentially useful for the treatment or prevention of Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas, the methods comprising: (a) administering a candidate drug to the transgenic animal of claim 7, and (b) detecting at least one of the following: (i) the suppression or prevention of expression of the protein coded for by the DNA molecule contained by said animal; or (ii) the increased degradation of the protein coded for by the DNA construct contained by said animal; due to the drug candidate compared to a control animal which has not received the candidate drug. Claim 15 specifies that the DNA construct contained by the animal has SEQ ID NO:1. Claim 16 specifies that the protein coded for by the DNA construct contained by the animal is over-expressed in the

brain of the animal. Support for claims 14-16 can be found throughout the specification, for example, at page 21, lines 3-20, and in claims 14-16 as originally filed.

Claims 37 and 38 are directed to *in vivo* methods for screening a candidate drug that is potentially useful for the treatment or prevention of Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas, the methods comprising: (a) administering a candidate drug to the transgenic animal of claim 7, wherein the transgenic animal exhibits at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons; and (b) detecting the reduction of frequency of at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons in the host due to the drug candidate compared to a control animal which has not received the candidate drug. Claim 38 specifies that the DNA construct contained by the animal has SEQ ID NO:1. Support for claims 37 and 38 can be found throughout the specification, for example, at page 20, line 26 through page 21, line 20, and in claims 14 and 15 as originally filed.

VI. Issues on Appeal (37 C.F.R. § 1.192(c)(6))

A. Written Description

The first issue on appeal is whether claims 7, 9, 14, 16, 35 and 36 are unpatentable under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. *See* Paper No. 16, page 2, line 20, through page 3, line 2.

B. Enablement

The second issue on appeal is whether claims 7-9, 14-16 and 35-40 are unpatentable under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. *See* Paper No. 16, page 6, lines 14-17.

VII. Grouping of Claims (37 C.F.R. § 1.192(c)(7))

For the purpose of this appeal, the pending claims do not stand or fall together.

On the issue of written description, the claims are grouped as follows:

Group 1: Claims 7, 9, 14, 16, 35, 36, 37, 39 and 40; and

Group 2: Claims 8, 15 and 38.

On the issue of enablement, the claims are grouped as follows:

Group 3: Claims 7, 9, 14, 16, 35, 36, 39 and 40;

Group 4: Claims 8 and 15;

Group 5: Claim 37; and

Group 6: Claim 38.

An explanation for the grouping of the claims is provided in the Argument section, immediately below.

VIII. Argument (37 C.F.R. § 1.192(c)(8))

A. Explanation for the Grouping of the Claims

1. Written Description

The written description rejection is based on the Examiner's contention that DNA molecules that are at least 90% homologous to SEQ ID NO:1 are not adequately described in the specification. *See* Paper No. 16, pages 2-6. Only claims 7, 9, 14, 16, 35, 36, 37, 39 and 40 (Group 1) encompass or include the use of transgenic animals containing a DNA molecule which is at least 90% homologous to SEQ ID NO:1. Claims 8, 15 and 38 (Group 2) specify that the DNA molecule contained by the transgenic animal has SEQ ID NO:1. Thus, the rejection for alleged insufficient description should not apply to the claims of Group 2.

2. Enablement

The Examiner has set forth two separate bases for the enablement rejection. First, the Examiner asserted that it would have required undue experimentation for a person of ordinary skill in the art to obtain a DNA molecule that is at least 90% homologous to SEQ ID NO:1 and that codes for a protein that has an activity of AD7c-NTP when over-expressed in neuronal cells. *See* Paper No. 16, pages 7-8. Second, the Examiner asserted that the specification does not enable the production of transgenic animals that exhibit a specific phenotype. *See* Paper No. 16, pages 9-14.

Claims 7, 9, 14, 16, 35, 36, 39 and 40 (Group 3) encompass or include the use of transgenic animals containing a DNA molecule which is at least 90% homologous to SEQ ID NO:1, but *do not* specify any phenotype exhibited by the transgenic animals. Thus, the Examiner's second basis of the rejection should not apply to the claims of Group 3.

Claims 8 and 15 (Group 4) encompass or include the use of transgenic animals containing a DNA molecule having SEQ ID NO:1, and *do not* specify any phenotype exhibited by the transgenic animals. Thus, neither basis for the enablement rejection can properly apply to the claims of Group 4.

Claim 37 (Group 5) encompasses methods that include the use of transgenic animals containing a DNA molecule which is at least 90% homologous to SEQ ID NO:1, and specify that the transgenic animals exhibit at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons.

Claim 38 (Group 6) encompasses methods that include the use of transgenic animals containing a DNA molecule having SEQ ID NO:1, and specify that the transgenic animals exhibit at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons. Thus, the Examiner's first basis of the rejection should not apply to the claim of Group 6.

B. Written Description

1. Legal Standard for Written Description

To satisfy the written description requirement of 35 USC § 112, first paragraph, an Applicant must convey with reasonable clarity to those skilled in the art that, as of the

effective filing date, the Applicant was in possession of the invention. *See Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). As made clear by the Federal Circuit, "[t]he written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed.'" *Union Oil Co. of Cal. v. Atlantic Richfield Co.*, 208 F.3d 989, 997, 54 USPQ2d 1227, 1232 (Fed. Cir. 2000).

The Federal Circuit has recently adopted the standard for determining compliance with the written description requirement as set forth in the USPTO's "Guidelines for the Examination of Patent Applications under 35 U.S.C. § 112, first paragraph, Written Description Requirement." *See Enzo Biochem, Inc. v. Gen-Probe Inc.*, 296 F.3d 1316, 1324, 63 USPQ2d 1609, 1613 (Fed. Cir. 2002). According to the USPTO's Guidelines:

> An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.

MPEP § 2163; *See also, Enzo*, 296 F.3d at 1324, 63 USPQ2d at 1613.

2. The Claimed Invention is More Than Adequately Described in the Specification

The written description rejection is not based on the sufficiency of description provided for the claimed transgenic animals *per se*; rather, the rejection is based on the Examiner's contention that DNA molecules that are at least 90% homologous to SEQ ID

NO:1 are not adequately described in the specification. *See* Paper No. 16, pages 2-6. Only claims 7, 9, 14, 16, 35, 36, 37, 39 and 40 (Group 1) encompass or include the use of transgenic animals containing a DNA molecule which is at least 90% homologous to SEQ ID NO:1. Appellants submit that the written description requirement of 35 U.S.C. § 112, first paragraph, is fully satisfied for the DNA molecules included within the transgenic animals of the claims of Group 1.

First, the DNA molecules included within the germ and somatic cells of the transgenic animals are not defined *solely* in terms of their function. As specified in claim 7, the germ and somatic cells of the transgenic animals comprise the DNA molecule *of SEQ ID NO: 1* or a DNA molecule *which is at least 90% homologous thereto*. Thus, the claims include a structural definition of the DNA constructs.

Second, procedures for isolating nucleic acid molecules that are at least 90% homologous to SEQ ID NO:1 are described in the specification and were well-known in the art. *See, e.g.*, Specification at page 19, lines 3-15. Moreover, assays are described in the specification for determining whether a DNA molecule encodes a protein having an activity of AD7c-NTP when over-expressed in neuronal cells. *See* Specification at page 20, line 1, through page 21, line 2. The specification also provides a working example that describes and illustrates the neuronal abnormalities that are caused by over-expressing AD7c-NTP in neuronal cells. *See* Specification at page 46, lines 1-26 and Figs. 6A-6G.

The detail provided in the specification for obtaining DNA molecules that are at least 90% homologous to SEQ ID NO: 1 and for determining whether they encode proteins having an activity of AD7c-NTP when overexpressed in neuronal cells would indicate to persons of ordinary skill in the art that Applicants were in possession of DNA molecules

having a nucleotide sequence that is at least 90% identical to SEQ ID NO: 1. By extension, persons of ordinary skill in the art would have recognized that Applicants had invented transgenic animals whose germ and somatic cells comprise a DNA molecule that is at least 90% homologous to SEQ ID NO:1, and screening methods involving the use thereof.

3. Errors in the Rejection

The Examiner has asserted that the specification does not provide sufficient description of a genus of DNA molecules with 90% homology to SEQ ID NO: 1 that code for proteins that have an activity of AD7c-NTP when over-expressed in neuronal cells. *See* Paper No. 16, page 3, line 21, through page 4, line 1. To support the rejection, the Examiner stated that:

> The as-filed specification fails to provide the essential nucleotide or amino acid residues for a representative number of sequences, wherein each sequence is composed of [sic] at least 90% homologous to SEQ ID NO: 1, that has an activity of AD7c-NTP when expressed (under expression, normal expression, etc.) in neuronal cells, wherein said overexpression of said sequence results in the reduction of frequency of at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons in said cells.

Paper No. 16, page 5, lines 12-18.

The Examiner has not demonstrated that Appellants have failed to comply with the written description requirement. The Examiner has based the written description rejection on the supposed absence from the specification of "[t]he essential nucleotides required for an activity of AD7c-NTP." Paper No. 16, page 3, lines 19-20. Although the specification does not identify any particular nucleotides of SEQ ID NO:1 as being "essential," the specification nonetheless provides a thorough description of the sequence characteristics and

motifs found in the amino acid sequence encoded by SEQ ID NO:1. In particular, the specification and drawings show (1) the hydrophobic leader sequence, (2) the myristoylation motif, (3) the potential AI cleavage site, (4) the region of homology with the insulin/IGF-1 chimeric receptor, (5) the potential glycogen synthase kinase-3, protein kinase C and cAMP or Ca-dependent kinase II phosphorylation motifs, (6) the transforming growth factor motif, (7) the sequences that exhibit significant homology with the A4 alternatively spliced mutant form of NF2, the β subunit of integrin and the human decay accelerating factor 2 precursor, and (8) the sequences that exhibit significant homology with human integral membrane protein and myelin oligoglycoprotein-16. *See* Specification at page 7, line 21, through page 8, line 3. From this information, and the knowledge in the art for producing DNA molecules having a specified degree of homology to a reference sequence, a person of ordinary skill in the art would recognize that Appellants were in possession of the invention insofar as it relates to DNA molecules that are at least 90% homologous to SEQ ID NO:1. *See also* Section VIII.B.2 (pages 9-11 of this Brief). Likewise, a person of ordinary skill in the art would conclude that Appellants were in possession of transgenic animals whose cells comprise a DNA molecule that is at least 90% homologous to SEQ ID NO:1, and of screening methods that involve the use of such transgenic animals. *See id.*

The USPTO's Synopsis of Application of Written Description Guidelines (hereinafter "Written Description Synopsis,"[1]) specifically supports Appellants' assertion that the written description requirement is satisfied for the claims of Group 1. Example 14 of the Written Description Synopsis (copy attached hereto as Exhibit 1) involves an analysis of the following claim: "A protein having SEQ ID NO:3 and variants thereof that are at least 95%

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[1]Copy available at http:// www.uspto.gov/web/menu/written.pdf

identical to SEQ ID NO:3 and catalyze the reaction of A→B."[2] Written Description Synopsis at page 53. The specification supporting this claim provides the following information:

> The specification exemplifies a protein isolated from liver that catalyzes the reaction of A→B. The isolated protein was sequenced and was determined to have the sequence as set forth in SEQ ID NO:3. The specification also contemplates but does not exemplify variants of the protein wherein the variant can have any or all of the following: substitutions, deletions, insertions and additions. The specification indicates that procedures for making proteins with substitutions, deletions, insertions and additions is routine in the art and provides an assay for detecting the catalytic activity of the protein.

Written Description Synopsis at page 53.

The Written Description Synopsis, Example 14, concludes that the disclosure meets the requirements of 35 USC § 112, first paragraph, in part because "procedures for making variants of SEQ ID NO:3 which have 95% identity to SEQ ID NO:3 and retain its activity are conventional in the art." *See id.* Moreover, it is noted that:

> [t]he single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO:3 which are

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[2]Appellants note that Example 14 of the Written Description Synopsis involves an analysis of a claim directed to a *protein molecule*, whereas Appellants' claims are directed to *transgenic animals* and *screening methods* using transgenic animals. Nevertheless, the present claims include or involve the use of the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto. In addition, the Examiner's first basis for the written description rejection is that "the specification does not provide sufficient description of a genus of DNA molecules with 90% homology to SEQ ID NO:1 . . ." Thus, the guidance provided by Example 14 of the Written Description Synopsis is directly relevant to the issue of whether sufficient written description is provided for Appellants' claims.

> capable of the specified catalytic activity. One of skill in the art would conclude that applicant was in possession of the necessary common attributes possessed by the members of the genus.

Written Description Synopsis at pages 54-55.

The situation presented in Example 14 of the Written Description Synopsis closely parallels the circumstances surrounding the DNA molecules that are used with or are included within the subject matter of Appellants' claims and the written description provided therefor. As such, Appellants submit that the guidance and instructions provided by the USPTO for analyzing a claim for compliance with the written description requirement strongly supports Appellants' assertion that the written description requirement of § 112, first paragraph, is satisfied for Appellants' claims.

First, in Example 14, it is stated that "all variants [encompassed by the claim] must possess the specified catalytic activity and must have at least 95% identity to the SEQ ID NO:3." Written Description Synopsis at page 54. Similarly, all of the species of DNA construct used with, or found within, the claims of Group 1 must have at least 90% homology to SEQ ID NO:1 and must code for a protein having an activity of AD7c-NTP when over-expressed in neuronal cells.

Second, it is noted in Example 14 that "[t]here is a single species disclosed, that species being SEQ ID NO:3," and that "[t]here is actual reduction to practice of the single disclosed species." Written Description Synopsis at page 54. Likewise, Appellants have disclosed SEQ ID NO:1 in the specification and have shown actual reduction to practice of SEQ ID NO:1. *See* specification at page 33, line 9, through page 35, line 28 (describing the isolation of the AD7c-NTP cDNA and the characteristics of the molecule); *see also* Figs. 1A-1C.

Third, according to Example 14, "procedures for making variants of SEQ ID NO:3 which have 95% identity to SEQ ID NO:3 and retain its activity are conventional in the art." Written Description Synopsis at page 53. Likewise, procedures for making DNA molecules which are at least 90% homologous to SEQ ID NO:1 and which encode proteins that retain the activity of AD7c-NTP are conventional in the art. As stated in the specification, DNA molecules which are at least 90% homologous to SEQ ID NO:1 may be isolated from cDNA libraries of humans and animals by hybridization under stringent conditions to the DNA molecule of SEQ ID NO:1 according to methods known to those of skill in the art. *See* Specification at page 19, lines 3-15. Appellants note that many other methods for obtaining DNA molecules that are included within the presently claimed invention were well known to persons having ordinary skill in the art at the time of the invention; examples include random and directed mutagenesis of a DNA molecule to produce a variant of SEQ ID NO:1 that is at least 90% homologous thereto. *See* Section VIII.C.2(a)(i) below (pages 21-23 of this Brief). In addition, proteins encoded by variants of SEQ ID NO:1 can easily be tested for AD7c-NTP activity using the procedures described in the specification as well as with other methods that are conventional in the art for testing the biological activity of a protein.

Fourth, in Example 14 of the Written Description Synopsis, it is stated that "an assay is described [in the specification] which will identify other proteins having the claimed catalytic activity." Written Description Synopsis at page 53. Correspondingly, in Appellants' specification, assays are described which will identify other DNA molecules encoding proteins having an activity of AD7c-NTP. For example, the specification describes the production of transgenic animals which over-express AD7c-NTP and the analysis of such animals for "evidence of neuronal or neuritic abnormalities associated with

Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas and gliblastomas." *See* Specification at page 20, lines 1-29. (The ability of one skilled in the art to produce transgenic animals is described in more detail in Sections VIII.C.2(a)(ii) and (c) below (pages 23-24 and 26-29 of this Brief)). The specification also provides an example of an *in vitro* assay for AD7c-NTP activity involving the over-expression of AD7c-NTP in neuronal cells and the analysis of such cells for growth properties and morphology, including the occurrence of apoptosis and neuritic sprouting. *See* Specification at page 45, line 16, through page 46, line 26, and Figs. 6A-6G.

As demonstrated above, the hypothetical situation described in Example 14 of the USPTO's Written Description Synopsis is very similar to the situation presented for the DNA molecules used with, or found within the subject matter encompassed by the claims of Group 1. Since the USPTO guidelines conclude that adequate written description is provided for the hypothetical claim in Example 14, it follows that there is adequate written description for the DNA molecules used with, or found within, the subject matter of Appellants' claims.

In *Enzo*, the Federal Circuit also made specific reference to Example 9 of the Written Description Synopsis. *See Enzo*, 296 F.3d at 1328, 63 USPQ2d at 1615. The analysis set forth in Example 9 (copy attached hereto as Exhibit 2) further supports Appellants' contention that the written description requirement is satisfied for the claims involved in this appeal and that the rejection was in error. Example 9 involves an analysis of the written description provided for a claim to "[a]n isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO: 1, wherein said nucleic acid encodes a protein that binds to a dopamine receptor and

stimulates adenylate cyclase activity." Written Description Synopsis at pages 35-36. According to the Example, there is a single species disclosed, *i.e.*, SEQ ID NO: 1. *See* Written Description Synopsis at page 35.

It is concluded that the written description requirement is satisfied for the hypothetical claim of Example 9. *See* Written Description Synopsis at page 37. The Example notes that "hybridization techniques using a known DNA as a probe under highly stringent conditions were conventional in the art at the time of filing." Written Description Synopsis at page 36. Similarly, Appellants' claims recite "a DNA molecule which is at least 90% homologous [to SEQ ID NO:1]." Techniques for identifying DNA molecules that are at least 90% homologous to a reference nucleic acid sequence were conventional in the art at the time of the effective filing date of the present application. *See* Specification at page 19, lines 3-15. As a specific exemplary method for isolating DNA molecules that are at least 90% homologous to SEQ ID NO: 1, the specification describes "hybridization under stringent conditions." Specification at page 19, line 5. The specification further notes:

> Stringent hybridization conditions are employed which select for DNA molecules having at least 40%, 85% and 90% homology to Seq. ID No. 1 are described in Sambrook *et al.*, In: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); and Maniatis *et al.*, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1985. The hybridizations may be carried out in 6 x SSC/5 x Denhardt's solution/0.1% SDS at 65°C. The degree of stringency is determined in the washing step. Thus, suitable conditions include 0.2 x SSC/0.01% SDS/65°C and 0.1 x SSC/0.01% SDS/65°C.

Specification at page 19, lines 6-15.

The analysis provided in Example 9 of the Written Description Synopsis further states that:

> a person of skill in the art would not expect substantial variation among species encompassed within the scope of the claims because the highly stringent hybridization conditions set forth in the claim yield structurally similar DNAs. Thus, a representative number of species is disclosed, since highly stringent hybridization conditions in combination with the coding function of DNA and the level of skill and knowledge in the art are adequate to determine that applicant was in possession of the claimed invention.

Written Description Synopsis at pages 36-37.

Analogously, a person of ordinary skill in the art would not expect substantial variation among species of DNA molecules included within the transgenic animals of Appellants' claims because the 90% homology to SEQ ID NO:1, specified in the claims, would yield "structurally similar DNAs." Based on the reasoning set forth in Example 9 of the Written Description Synopsis, it must be concluded that a representative number of species is disclosed because 90% homology to SEQ ID NO:1 in combination with the coding function of DNA and the level of skill and knowledge in the art are adequate to determine that Appellants were in possession of the claimed invention.

Accordingly, Appellants respectfully request that the Board reverse the Examiner's § 112, first paragraph rejection for alleged insufficient written description and remand this application for issue.

C. Enablement

1. Legal Standard for Enablement

In order to satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph, Appellants' specification must enable any person skilled in the art to make and use the

claimed invention without undue experimentation. *See In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). *See also United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988). The factors to be considered when determining whether the necessary experimentation is "undue" include: (a) the breadth of the claims, (b) the nature of the invention, (c) the state of the prior art, (d) the level of one of ordinary skill, (e) the level of predictability in the art, (f) the amount of direction provided by the inventor, (g) the existence of working examples, and (h) the quantity of experimentation needed to make or use the invention based on the content of the disclosure. *See Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. Moreover, as long as the specification discloses at least one method for making and using the claimed invention, then the enablement requirement of 35 U.S.C. § 112, first paragraph is satisfied. *See Johns Hopkins Univ. v. CellPro, Inc.*, 152 F.3d 1342, 1361, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998).

An Applicant is not limited to the confines of the specification to provide the necessary information to enable an invention. *See In re Howarth*, 654 F.2d 103, 105-6, 210 USPQ 689, 692 (CCPA 1981). An Applicant need not supply information that is well known in the art. *See Genentech, Inc. v. Novo Nordisk*, 108 F.3d 1361, 1366, 42 USPQ2d 1001, 1005 (Fed. Cir. 1997); *Howarth*, 654 F.2d at 105-6, 210 USPQ at 692; *see also In re Brebner*, 455 F.2d 1402, 173 USPQ 169 (CCPA 1972) (finding a disclosure enabling because the procedure for making the starting material, although not disclosed, would have been known to one of ordinary skill in the art as evidenced by a Canadian patent). "'That which is common and well known is as if it were written out in the patent and delineated in the drawings.'" *Howarth*, 654 F.2d at 106, 210 USPQ at 692 (quoting *Webster Loom Co. v. Higgins* et al., 105 U.S. (15 Otto.) 580, 586 (1881)). Moreover, one of ordinary skill in

the art is deemed to know not only what is considered well known in the art but also where to search for any needed starting materials. *See Id.*

In order to establish a *prima facie* case of lack of enablement, the Examiner has the initial burden to set forth a reasonable basis to question the enablement provided for the claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). To satisfy this burden, "it is incumbent upon the Patent Office. . . to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *See In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971) (emphasis in original). As enunciated by the Federal Circuit:

> [A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

In re Brana, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (emphasis in original; quoting *Marzocchi*, 439 F.2d at 224, 169 USPQ at 370).

2. The Claimed Invention is Fully Enabled

(a) The Subject Matter of The Claims of Group 3 (Claims 7, 9, 14, 16, 35, 36, 39 and 40) is Fully Enabled

Claims 7, 9, 35, 36, 39 and 40 are directed to transgenic non-human animals, all of whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA

molecule which is at least 90% homologous thereto, wherein said DNA molecule is expressed in one or more cells of said transgenic animal, and wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when over-expressed in neuronal cells. Claims 14 and 16 are directed to *in vivo* methods for screening a candidate drug that is potentially useful for the treatment or prevention of Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas, comprising, *inter alia*, administering a candidate drug to the transgenic animal of claim 7. A person of ordinary skill in the art would have been able to make and use the transgenic non-human animals of the invention, and practice the claimed methods, using routine methods in the art.

(i) Obtaining a DNA Molecule of SEQ ID NO:1 or a DNA Molecule Which is at Least 90% Homologous Thereto

A person of ordinary skill in the art would have been able to obtain the DNA molecules that are used to produce the transgenic animals of the claims of Group 3. More specifically, a person of ordinary skill in the art, in view of the present specification, would have been able to obtain a DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, wherein the DNA molecule codes for a protein that has an activity of AD7c-NTP when over-expressed in neuronal cells, using only routine methods in the art.

The specification provides exemplary methods for obtaining DNA molecules which are at least 90% homologous to SEQ ID NO:1. Such methods involve the isolation of DNA molecules from cDNA libraries by hybridization under stringent conditions to the DNA molecule of SEQ ID NO:1. *See* specification at page 19, lines 3-15. Additional methods

for obtaining DNA molecules that are at least 90% homologous to SEQ ID NO:1 include the use of directed and random mutagenesis techniques. Such methods were well known to those of ordinary skill in the art at the time of the invention. *See, e.g.*, Sambrook *et al.*, "Creating Many Mutations in a Defined Segment of DNA," in *Molecular Cloning, A Laboratory Manual*, Sambrook *et al.*, eds., Cold Spring Harbor Laboratory Press, pp. 15.95-15.108 (1989) (copy attached herewith as Exhibit 3).

Once obtained, DNA molecules that are at least 90% homologous to SEQ ID NO:1 could have easily been tested for the ability to encode a protein having an activity of AD7c-NTP. The specification describes various methods for assaying for AD7c-NTP activity. For example, transgenic animals can be made that over-express AD7c-NTP in neuronal cells, and, once obtained, the transgenic animals may be analyzed for evidence of neuronal or neuritic abnormalities associated with Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas and glioblastomas. *See* specification at page 20, lines 1-29. (The ability of a person of ordinary skill in the art to produce transgenic animals whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto is discussed in Sections VIII.C.2(a)(ii) and (c) below (pages 23-24 and 26-29 of this Brief)).

Additionally, *in vitro* methods can be used to test for AD7c-NTP activity. For example, the specification exemplifies an assay involving the overexpression of AD7c-NTP in neuronal cells and the subsequent analysis for cellular characteristics of Alzheimer's disease, including apoptosis and neuritic sprouting. *See* specification at page 46, lines 4-26. Thus, the full range of DNA molecules that are included within the transgenic animals of

the claims of Group 3 could have been easily made and analyzed by persons of ordinary skill in the art using only routine methods and experimentation.

(ii) Creating Transgenic Non-Human Animals

A person of ordinary skill in the art, using routine methods in view of the present specification, would also have been able to create transgenic non-human animals, all of whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, and wherein the DNA molecule is over-expressed in one or more cells of the transgenic animal.

The specification describes exemplary methods for obtaining transgenic animals including, *e.g.*, injecting a DNA construct into a fertilized egg which is allowed to develop into an adult animal, and generating transgenic animals with embryonic stem cell technology. *See* specification at page 20, lines 3-17. The specification also provides several references which describe additional methods of preparing transgenic animals. *See* specification at page 20, lines 18-25. For example, the specification at page 20, line 19, cites U.S. Patent No. 5,602,299 (hereinafter "Lazzarini") (incorporated by reference in the specification in its entirety). Lazzarini states:

> Any technique known in the art may be used to introduce the transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection; retrovirus mediated gene transfer into germ lines; gene targeting in embryonic stem cells; electroporation of embryos; and sperm-mediated gene transfer; etc.

See U.S. Patent No. 5,602,299 at column 13, lines 2-12, internal citations omitted. *See also*, references cited in Section VIII.C.2(c) (pages 26-29 of this Brief), which illustrate several instances of the successful production of transgenic animals.

Thus, it would have required only routine experimentation to create and use the transgenic animals encompassed by claims 7, 9, 35, 36, 39 and 40.

(iii) In Vivo Screening Methods

Claims 14 and 16 are directed to *in vivo* methods for screening a candidate drug that is potentially useful for the treatment or prevention of Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas. The methods of claims 14 and 16 comprise the use of the transgenic animal encompassed by claim 7. A person of ordinary skill in the art would have been able to practice the screening methods encompassed by claims 14 and 16 with only routine experimentation.

As demonstrated in Sections VIII.C.2(a)(i) and (ii) (pages 21-24 of this Brief), it would have required only routine experimentation for a skilled artisan to obtain transgenic animals encompassed by claim 7. Moreover, the administration of a candidate drug to the transgenic animal of claim 7 would have been a matter of routine procedure in the art.

In addition, a person of ordinary skill in the art could have performed the detecting aspects of claims 14 and 16 with only routine experimentation. Specifically, detecting: (i) the suppression or prevention of expression of the protein coded for by the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, and (ii) the increased degradation of the protein, would have been a matter of routine procedure in the art. As discussed in the specification, the suppression or prevention of expression, and the

increased degradation of the protein, could have been detected, for example, with antibodies specific for AD7c-NTP. *See* Specification at page 21, lines 21-23. As noted in the specification:

> Monoclonal and polyclonal antibodies which are specific for AD7c-NTP as well as methods for the qualitative and quantitative detection of AD7c-NTP are described herein as well as in WO94/23756 and U.S. appl. no. 08/340,426. Such testing may be carried out on CSF of the transgenic animal or by immunohistochemical staining of a tissue section from the brain of the animal. In addition, such testing may be carried out by Western blot analysis, ELISA or RIA.

Specification at page 21, lines 23-29. Other methods for monitoring protein expression, such as RT-PCR and *in situ* hybridization, would have been available to one of ordinary skill in the art as well. *See* Specification at page 42, line 1, through page 43, line 24.

In view of the teachings of the specification and drawings and the knowledge possessed by those of ordinary skill in the art, it would have required only routine experimentation to practice the full scope of the methods encompassed by claims 14 and 16.

(b) The Subject Matter of The Claims of Group 4 (Claims 8 and 15) is Fully Enabled

Claim 8 is directed to transgenic non-human animals, all of whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1, wherein the DNA molecule is over-expressed in one or more cells of the transgenic animal, and wherein the DNA molecule codes for a protein that has an activity of AD7c-NTP when over-expressed in neuronal cells. As discussed above, a person of ordinary skill in the art would have been able to make and use the transgenic animals of the invention, all of whose germ and somatic cells comprise a DNA molecule that is at least 90% homologous to SEQ ID NO:1, using only routine

methods in the art. *See* Sections VIII.C.2(a)(i) and (ii) (pages 21-24 of this Brief). It therefore follows that a person of ordinary skill in the art would have been able to make and use transgenic animals, all of whose germ and somatic cells comprise a DNA molecule having SEQ ID NO:1, using only routine methods. Accordingly, the subject matter of claim 8 is fully enabled.

Claim 15 is directed to the method of claims 14, wherein the DNA construct contained by the transgenic animal has SEQ ID NO:1. As discussed above, person of ordinary skill in the art would have been able to practice the methods of the invention involving the use of transgenic animals whose germ and somatic cells comprise a DNA molecule that is at least 90% homologous to SEQ ID NO:1 using only routine experimentation. *See* Section VIII.C.2(a)(iii) (pages 24-25 of this Brief). It therefore follows that a person of ordinary skill in the art would have been able to practice the methods of the invention involving the use of transgenic animals whose germ and somatic cells comprise a DNA molecule having SEQ ID NO:1, using only routine experimentation.

Accordingly, the subject matter of the claims of Group 4 is fully enabled.

(c) The Subject Matter of The Claim of Group 5 (Claim 37) is Fully Enabled

Claim 37 is directed to *in vivo* methods for screening a candidate drug that is potentially useful for the treatment or prevention of Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas. The methods of claim 37 comprise, *inter alia*, administering a candidate drug to the transgenic animal of claim 7, wherein the transgenic animal exhibits at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons. A person of

ordinary skill in the art would have been able to practice the methods of claim 37 using only routine experimentation.

As demonstrated in Sections VIII.C.2(a)(i) and (ii) (pages 21-24 of this Brief), it would have required only routine experimentation for a skilled artisan to obtain transgenic animals encompassed by claim 7. Moreover, it would have only required routine experimentation to obtain a transgenic animal of claim 7, wherein the transgenic animal exhibits at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons.

The specification describes exemplary methods for obtaining transgenic animals and cites several references describing additional methods of preparing transgenic animals. *See* Specification at page 20, lines 3-25. These methods would have guided a skilled artisan in producing the transgenic animals used in the methods of claim 37.

In addition to the teachings of the specification, the knowledge available in the art would have provided additional guidance for the production of the transgenic animals used in the methods of claim 37. Several examples from the scientific literature (available as of the effective filing date of the present application) demonstrate the successful production of transgenic animals that exhibited particular phenotypes caused by the presence of the transgene. *See* Appellants' Amendment and Reply Under 37 C.F.R. § 1.111, filed August 8, 2002 at pages 31-32. These references describe: (i) the production of transgenic rats that expressed the human HLA-B27 gene and that exhibited a spontaneously arising disease that "showed a striking clinical and histologic similarity to B27-associated disease in humans," *see* Hammer, R.E. *et al.*, *Cell 63*:1099-1112 (1990) (included herewith as Exhibit 4), *see* especially page 1108, right column, bottom paragraph; (ii) the production of transgenic rats

that expressed an additional rat *Ren-2* gene and that exhibited hypertension and associated phenotypes, *see* Lee, M.A. *et al.*, *Am. J. Physiol. 270*:E919-E929 (1996) (included herewith at Exhibit 5), *see* especially page E921, left column, first full paragraph; (iii) the production of transgenic mice and a transgenic pig that expressed the human complement inhibitor hCD59, the cells of which exhibited resistance to challenge with high-titer anti-porcine antibody and human complement, *see* Fodor, W.L. *et al.*, *Proc. Natl. Acad. Sci. USA 91*:11153-11157 (1994) (included herewith as Exhibit 6), *see* especially page 11157, left column, last full paragraph; and (iv) the production of a transgenic pig that expressed a murine leukemia virus-rat somatotropin fusion gene and that produced high levels of circulating rat somatotropin and exhibited increased skeletal growth and reduced fat deposition, *see* Ebert, K.M. *et al.*, *Mol. Endocrinol. 2*:277-283 (1988) (included herewith as Exhibit 7), *see* especially paragraph bridging pages 280-281. These references demonstrate that producing transgenic animals having particular phenotypes would not have required undue experimentation.

The scientific literature also provides examples of transgenic animals that specifically exhibit neurological phenotypes indicative of AD. *See* Appellants' Reply to Final Office Action, filed July 7, 2003 at page 15. Exemplary references include Quon *et al.*, *Nature 352*:239-241 (1991) (copy included herewith as Exhibit 8); Wirak *et al.*, *Science 253*:323-325 (1991) (copy included herewith as Exhibit 9); Kammesheidt *et al.*, *Proc. Natl. Acad. Sci. USA 89*:10857-10861 (1992) (copy included herewith as Exhibit 10); Games *et al.*, *Nature 373*:523-527 (1995) (copy included herewith as Exhibit 11); and Johnson-Wood *et al.*, *Proc. Natl. Acad. Sci. USA 94*:1550-1555 (1997) (copy included herewith as Exhibit

12). These references provide further evidence that the transgenic animals used in the methods of claim 37 could have been obtained without undue experimentation.

A person of ordinary skill in the art could also have performed the detecting aspects of claim 37 with only routine experimentation. That is, detecting the reduction of frequency of at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons in the host would have required only routine experimentation. As noted in the specification, the frequency of these phenotypes could have been determined easily using, *e.g.*, immunohistochemical staining. *See* Specification at page 22, lines 1-12. The *in vitro* results presented in the specification relating to AD7c-NTP over-expression would have provided additional guidance for one skilled in the art to detect the reduction of frequency of at least one of the recited cellular phenotypes. *See, e.g.*, specification at page 46, lines 1-26 and Figs. 5 and 6A-6G.

Accordingly, the subject matter of claim 37 is fully enabled.

(d) The Subject Matter of The Claim of Group 6 (Claim 38) is Fully Enabled

Claim 38 is directed to the methods of claim 37, wherein the DNA construct contained by the transgenic animal has SEQ ID NO:1. As discussed above, a person of ordinary skill in the art would have been able to practice the methods of claim 37, which involve the use of transgenic animals whose germ and somatic cells comprise a DNA molecule that is at least 90% homologous to SEQ ID NO:1, using only routine experimentation. *See* Section VIII.C.2(c) (pages 26-29 of this Brief). It therefore follows that a person of ordinary skill in the art would have been able to practice the methods of the invention involving the use of transgenic animals whose germ and somatic cells comprise

a DNA molecule having SEQ ID NO:1 using only routine experimentation. Accordingly, the subject matter of claim 38 is fully enabled.

3. Errors in the Rejection

The Examiner has not set forth a reasonable basis to question the enablement provided for the claimed invention. Thus, a *prima facie* case of non-enablement has not been established. *See Wright*, 999 F.2d at 1562, 27 USPQ2d at 1513; *Marzocchi*, 439 F.2d at 224, 169 USPQ at 370.

(a) Errors Related to the Enablement Provided for DNA Molecules that are At Least 90% Homologous to SEQ ID NO:1

The rejection for lack of enablement is based, in part, on the assertion that it would have required undue experimentation for a skilled artisan to obtain a DNA molecule that is at least 90% homologous to SEQ ID NO:1 and that codes for a protein that has an activity of AD7c-NTP when over-expressed in neuronal cells. *See* Paper No. 16, pages 7-8. As discussed above, it would have required only routine experimentation for a skilled artisan to obtain the DNA molecules that are used to produce the transgenic animals of the invention. *See* Section VIII.C.2(a)(i) (pages 21-23 of this Brief). In addition, the Examiner has not presented sufficient evidence or scientific reasoning to support the rejection.

The Examiner stated that "[t]he specification does not disclose which nucleotides of the claimed DNA molecule [are] considered essential for one skilled in the art to make a representative number of DNA molecules with 90% homology to SEQ ID NO:1." Paper No. 16, page 7, lines 17-20. The Examiner, however, has not explained why it is believed that

the production of DNA molecules that are included within the transgenic animals of the invention would require the identification of "essential" nucleotides. As discussed above, there are numerous methods that were available in the art at the time of the application that could have been used to produce DNA molecules that are 90% homologous to SEQ ID NO:1. *See* Section VIII.C.2(a)(i) (pages 21-23 of this Brief). Such methods do not require knowledge of "essential" nucleotides. So long as the expressed protein has an activity of AD7c-NTP, the coding nucleotides are useful in the present invention without knowing whether any of the nucleotides are "essential." Thus, the Examiner's statement regarding the identification of "essential" nucleotides does not support a rejection for lack of enablement.

The Examiner stated that "the specification does not provide sufficient guidance or factual evidence for one skilled in the art to determine without an undue amount of experimentation . . . if the nucleic acid sequence with at least 90 percent homology to SEQ ID NO: 1, would exhibit the same biological function of SEQ ID NO: 1 (observed activity when the sequence is over-expressed in neuronal cells)." Paper No. 16, page 7, line 22, through page 8, line 4. This is an incorrect statement. As discussed in Section VIII.C.2(a)(i) (pages 21-23 of this Brief), *in vitro* and *in vivo* methods for determining whether a given DNA molecule codes for a protein that has an activity of AD7c-NTP are set forth in the specification and would not have required undue experimentation. No evidence has been presented to indicate that it would have required undue experimentation to determine whether a given DNA molecule codes for a protein that has an activity of AD7c-NTP. Thus, a *prima facie* case of non-enablement cannot be established on this basis.

The Examiner stated that:

> [s]ince, the relationship between a sequence of a peptide and its tertiary structure (i.e. its activity) are not well understood and are not predictable . . . it would [have] required undue experimentation for one skilled in the art to arrive at other DNA molecules with 90% homology to SEQ ID NO: 1 and having SEQ ID NO: 1 activity when over-expressed in neuronal cells.

Paper No. 16, page 8, lines 4-10. The Examiner has not explained why it is believed that the production of DNA molecules within the transgenic animals of the invention would have required a skilled artisan to predict the relationship between the sequence of a peptide and its structure. A skilled artisan would not need to predict the structural and/or functional consequences of particular mutations or base changes in order to produce DNA molecules that are 90% homologous to SEQ ID NO:1 and that code for proteins having an activity of AD7c-NTP. To make the DNA molecules that are included within the transgenic animals of the present invention, a skilled artisan would only need to be able to: (a) obtain DNA molecules that are at least 90% homologous to SEQ ID NO:1, and (b) test them for the ability to encode proteins that possess AD7c-NTP activity. As discussed in Section VIII.C.2(a)(i) (pages 21-23 of this Brief), both of these processes would have been routine in the art.

Of course, it is possible that DNA molecule that are at least 90% homologous to SEQ ID NO:1 may not encode proteins with AD7c-NTP activity. The skilled artisan, however, would be able to easily identify and discard such inactive molecules. Screening for molecules that possess a particular activity is both common and routine in the biological arts. Experimentation, even complex experimentation, is not undue if the art typically engages in such experimentation. *See In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd. sub nom.*, *Massachusetts Institute of*

Technology v. A.B. Fortia, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985); *see also Wands*, 858 F.2d at 737, 8 USPQ2d at 1404.

Thus, any uncertainty that is associated with predicting protein function from sequence data is irrelevant in an analysis of the enablement of Appellants' claims. A skilled artisan may easily screen for DNA molecules that are at least 90% homologous to SEQ ID NO:1 and that encode proteins having AD7c-NTP activity when over-expressed in neuronal cells. Such screening, even if it resulted in the identification of molecule not having the desired activity, is routine. Thus, the Examiner's statements relating to the ability of a skilled artisan to predict the relationship between the sequence of a peptide and its structure do not support a rejection for lack of enablement.

The Examiner cited *Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.*, 927 F.2d 1200 (Fed. Cir. 1991) to support the enablement rejection. *See* Paper No. 16, page 8, lines 10-17. The claims at issue in *Amgen*, however, were directed to DNA sequences defined solely in functional terms.[3] Appellants' claims specify that the germ and somatic cells of the transgenic animals comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, and wherein the DNA molecule codes for a protein that has an activity of AD7c-NTP when over-expressed in neuronal cells. Therefore, the reasoning set forth in *Amgen* for finding the claims non-enabled cannot be used to support a rejection of the present claims for lack of enablement.

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[3]Claim 7 of U.S. Patent No. 4,703,008, the claim at issue in the enablement analysis of *Amgen*, is as follows: "A purified and isolated DNA sequence consisting essentially of a DNA sequence encoding a polypeptide having an amino acid sequence sufficiently duplicative of that of erythropoietin to allow possession of the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells, and to increase hemoglobin synthesis or iron uptake." *Amgen* at 1204.

(b) Errors Related to the Enablement Provided for the Transgenic Animals

The rejection for lack of enablement is also based on the Examiner's contention that the specification does not provide sufficient guidance for a skilled artisan to make and use transgenic animals having a specific phenotype. *See e.g.*, Paper No. 16, pages 9-14. As discussed above, a person of ordinary skill in the art, in view of the specification, would have been able to make and use the transgenic animals of the invention with only routine experimentation. *See* Sections VIII.C.2(a)(ii) and (c) (pages 23-24 and 26-29 of this Brief). In addition, the Examiner has not presented sufficient evidence or scientific reasoning to indicate that making and using the transgenic animals of the invention would have required undue experimentation.

(i) Claims 7-9, 14-16, 35, 36, 39 and 40 Do Not Require That The Transgenic Animals Exhibit a Specific Phenotype

The rejection for lack of enablement is based to a large extent on the interpretation of the claims as requiring that the transgenic animals "[express] the protein at a level sufficient to result in a specific phenotype." Paper No. 16, page 11, line 10. For example, the Examiner stated that:

> [w]hile the state of the art of transgenics is such that one of skill in the art would be able to produce [a] transgenic animal comprising a transgene of interest (e.g. SEQ ID NO: 1 or a sequence with 90% homology thereto); it is not predictable if the transgene would be expressed at a level and specificity sufficient to cause a particular phenotype.

Paper No. 16, page 11, lines 18-22. The only claims that specify a particular phenotype exhibited by the transgenic animals are claims 37 and 38. The specification does not

indicate that claims 7-9, 14-16, 35, 36, 39 and 40 should be interpreted to require that the transgenic animals exhibit any specific phenotype. Thus, the Examiner's justification for the enablement rejection can only apply to claims 37 and 38. Moreover, as discussed in Section VIII.C.2(c) (pages 26-29 of this Brief), the production of transgenic animals with a particular phenotype would not have required undue experimentation. Therefore, the rejection insofar as it relates to claims involving the use of transgenic animals that exhibit a particular phenotype is improper.

The Examiner's explanation for interpreting the claims as requiring that the transgenic animals exhibit a particular phenotype is provided in the following passage:

> Note that although the claimed transgenic animal is not limited to expression of the protein at a level resulting in a specific phenotype, with regard to the claims breadth, the standard under 35 U.S.C. 112, first paragraph, entails the determination of what claims recite and what the claims mean as a whole. In addition, when analyzing the enabled scope of the claims, the teachings of the specification are to be taken into account because the claims are to be given their broadest reasonable interpretation that is consistent with the specification. As such, the broadest interpretation of the claimed transgenic animal having cells, which harbor a recombinant nucleic acid that expresses the protein at a level sufficient to result in a specific phenotype (i.e., it is unknown what other purpose the transgenic animal would serve if the transgene (e.g., SEQ ID NO: 1 or a sequence with 90% homology thereto) is not expressed at a sufficient level for a resulting phenotype).

Paper No. 16, page 11, lines 3-13. The foregoing statements do not support the interpretation of the claims as requiring that the transgenic animals of the invention exhibit any particular phenotype.

The Examiner noted that an analysis under 35 U.S.C. § 112, first paragraph, requires determining "what the claims recite and what the claims mean as a whole." Paper No. 16,

page 11, lines 4-6. None of the claims, except for claims 37 and 38, specify that the transgenic animals exhibit any specific phenotype. Thus, an analysis of the claim language does not support the position that the transgenic animals of claims 7-9, 14-16, 35, 36, 39 and 40 must exhibit any particular phenotype.

The Examiner also noted that, in an analysis under 35 U.S.C. § 112, first paragraph, "the teachings of the specification are to be taken into account." Paper No. 16, page 11, lines 6-8. The Examiner, however, has not pointed to anything in the specification that would support his claim interpretation. That is, nothing in the specification is cited to support the interpretation of claims 7-9, 14-16, 35, 36, 39 or 40 as requiring that the transgenic animals exhibit a specified phenotype.

The Examiner also stated that "it is unknown what other purpose the transgenic animal would serve if the transgene (e.g., SEQ ID NO: 1 or a sequence with 90% homology thereto) is not expressed at a sufficient level for a resulting phenotype." Paper No. 16, page 11, lines 10-13. Appellants have repeatedly pointed out that transgenic animals encompassed by or included within the subject matter of claims 7-9, 14-16, 35, 36, 39 and 40 are useful in, *e.g.*, drug screening applications even if the animals do not exhibit any specific phenotypes. *See* Amendment and Reply Under 37 C.F.R. § 1.111, filed August 8, 2002 at page 27; Amendment and Reply Under 37 C.F.R. § 1.111, filed January 22, 2003 at pages 26-27; and Reply to Final Office Action, filed July 7, 2003, pages 9-11.

Briefly, candidate drugs can be administered to a transgenic animal whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto. Candidate drugs can be identified by their ability to cause, *e.g.*, the suppression or prevention of expression of the protein encoded by the DNA

molecule contained by the transgenic animal. *See* Specification at page 21, line 12. Alternatively, drugs can be identified on the basis of their ability to increase the degradation of the protein encoded by the DNA molecule contained by the transgenic animal. *See* Specification at page 21, line 13. Method claims 14-16 specifically comprise detecting at least one of the following: (i) the suppression or prevention of expression of the protein coded for by the DNA molecule contained by said animal; or (ii) the increased degradation of the protein coded for by the DNA construct contained by said animal. Thus, the only characteristic that the transgenic animals encompassed by or included within the subject matter of claims 7-9, 14-16, 35, 36, 39 and 40 need to possess in order to be useful for the contemplated screening methods is that they express the DNA molecule of SEQ ID NO:1 or a DNA molecule that is at least 90% homologous thereto.

Since it is unnecessary for the transgenic animals encompassed by or included within the subject matter of claims 7-9, 14-16, 35, 36, 39 and 40 to exhibit any particular phenotype, the Examiner's statements regarding the supposed unpredictability of producing transgenic animals with a particular phenotype are irrelevant in an analysis of the enablement of these claims. The Examiner has conceded that "one of skill in the art would be able to produce transgenic animal comprising a transgene of interest (e.g. SEQ ID NO:1 or a sequence with 90% homology thereto)." Paper No. 16, page 11, lines 19-20. Accordingly, it must be concluded that the subject matter of claims 7-9, 14-16, 35, 36, 39 and 40 is adequately enabled.

(ii) The Examiner Has Not Set Forth Sufficient Evidence to Indicate That Producing Transgenic Animals with a Specific Phenotype Would Have Required Undue Experimentation

Notwithstanding the fact that none of the claims, except claims 37 and 38, specify a phenotype exhibited by the transgenic animals, it is incorrect that the production of transgenic animals of the invention that exhibit a specific phenotype (*e.g.*, neuritic sprouting, nerve cell death, nerve cell degeneration, neurofibrillary tangles, and/or irregular swollen neurites) would have required undue experimentation. The Examiner has not presented sufficient evidence or reasoning to support the assertion that making and/or using transgenic animals whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, wherein the transgenic animals exhibit at least one of neuritic sprouting, nerve cell death, nerve cell degeneration, neurofibrillary tangles, and/or irregular swollen neurites, would have required undue experimentation.

The Examiner cited Polejaeva *et al.*, *Theriogenology 53*:117-126 (2000) (copy attached hereto as Exhibit 13) as support for the contention that "producing transgenic animals with a predictable phenotype was considered unpredictable." Paper No. 16, page 9, lines 7-19. The Examiner specifically cited Polejaeva at page 119. Here, Polejaeva begins by acknowledging that "[t]ransgenic animals *can be successfully produced in a number of species* including mice, rabbits, pigs, sheep, cattle and goats by the injection of the gene of interest into the pronucleus of a zygote." *See* Polejaeva at page 119, second full paragraph (emphasis added, internal citations omitted). Thus, the first sentence of the cited passage of Polejaeva directly refutes the assertion that transgenic animal production using pronuclear microinjection would entail undue experimentation.

Polejaeva goes on to describe the "limitations" that are associated with pronuclear injection. The "most profound limitation," mentioned by Polejaeva, is that DNA can only be added, not deleted or modified *in situ*. *See id.* Since the production of transgenic animals that are included within Appellants' claims only requires the addition of DNA molecules, this "most profound limitation" is inapplicable to Appellants' invention.

The other two limitations of pronuclear microinjection cited by Polejaeva are (a) the potential for random integration of foreign DNA, and (b) the possibility of creating mosaic animals. *See id.* In view of the potential for random integration into an animal's genome using pronuclear microinjection, the Examiner asserted that:

> . . . it would take one skilled in the art an undue amount of experimentation to reasonably extrapolate from random integration to determining if a DNA sequence set forth in SEQ ID NO:1 is inserted at the correct site and is expressed at a level sufficient enough to produce a phenotype in any transgenic non-human animal.

Paper No. 16, page 10, lines 2-5. This is an incorrect statement and is directly refuted by the numerous examples in the art of the successful production of transgenic animals having specific phenotypes. *See* Section VIII.C.2(c) (pages 26-29 of this Brief).

Aside from citing Polejaeva for the proposition that random integration and the production of mosaics may be a "limitation" in the practice of pronuclear microinjection to produce transgenic animals, the Examiner has not set forth any evidence that would suggest that this method would require an "undue amount of experimentation." The potential limitations associated with pronuclear microinjection only indicate that a certain level of experimentation may be needed to create transgenic animals using this technology. The necessity of some experimentation to practice a claimed invention does not render an

invention non-enabled as long as the quantity of experimentation needed is not regarded as undue. *See In re Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219 (CCPA 1976).

There is no indication that the experimentation needed to successfully obtain transgenic animals with a particular phenotype using pronuclear microinjection would be regarded as *undue*. Indeed, the fact that transgenic animals with desired phenotypes have been successfully produced using pronuclear microinjection indicates that the practice of this technology would not have been regarded as requiring undue experimentation. *See* Polejaeva at page 119, second full paragraph. In addition, others in the field have noted that:

> Pronuclear DNA injection has enabled the scientific community world wide to selectively add defined genes of choice into the germ line of laboratory as well as farm animals. Many experiments with transgenic animals confirmed that transgenesis can provide new insight into many aspects of mammalian life, development and diseases.

See Rülicke and Hübscher, *Exp. Physiol. 85*:589-601 (2000) (copy attached hereto as Exhibit 14) at page 597, left column, first full paragraph.

Moreover, the final two sentences in the cited paragraph of Polejaeva indicate that alternative methods are available for producing transgenic animals that may avoid the limitations of pronuclear injection:

> Somatic cell nuclear transfer [described on page 120 of Polejaeva] will eliminate this problem and accelerate transgenic herd or flock generation. In addition, transgenic sheep produced using this new technology require the use of fewer than half the animals needed for pronuclear microinjection.

See Polejaeva at page 119, second full paragraph. Therefore, Polejaeva indicates that somatic cell nuclear transfer -- a method that would have been available to one of ordinary skill in

the art as of the effective filing date of the application -- is another method that is likely to be successful in the production of transgenic animals with a particular phenotype.

The Examiner next cited Trojanowski and Lee, *Brain Pathology* 9:733-739 (1999) (copy attached hereto as Exhibit 15) (hereinafter "Trojanowski") for the proposition that "certain characteristic[s] can be produced in a test tube, [but] the conditions required are highly artificial and in vitro paradigms have limited utility as models of in vivo mechanisms of neurodegeneration." Paper No. 16, page 10, lines 8-11. Trojanowski, however, simply emphasizes the need in the art for the development of additional transgenic mouse models of filamentous brain lesions. *See* Trojanowski at page 733. Trojanowski does not indicate or suggest that the production of transgenic animals would have required undue experimentation. In fact, Trojanowski reports the successful production of transgenic mouse lines that over-expressed the tau protein and that exhibited "pre-tangle" tau pathology. *See* Trojanowski at page 736, left column. Therefore, Trojanowski does not support the position that the claimed invention is not enabled.

Even though Trojanowski describes the successful production of transgenic mice that exhibited a specific phenotype, the Examiner dismissed this evidence because the transgenic mice mentioned in Trojanowski showed pre-tangle tau pathology, but "no filamentous tau inclusions." *See* Paper No. 16, page 15, lines 15-22 (quoting Trojanowski at page 736, left column). The absence of filamentous tau inclusions in the brains of the transgenic mice mentioned in Trojanowski does not suggest that producing transgenic animals exhibiting specific phenotypes, in general, would have required undue experimentation. As noted by Trojanowski, "it must be emphasized that efforts to produce animal models of tau pathologies have been *very limited*." Trojanowski at page 736, left column (citations

omitted, emphasis added). In other words, Trojanowski suggests that the failure to produce transgenic mice with filamentous tau inclusions is due to the fact that insufficient effort had been put forth to produce such animals.

Importantly, Trojanowski also reports unpublished results showing that transgenic mice with filamentous intraneuronal inclusions composed of tau and neurofilaments had in fact been produced:

> Encouraged by these results, and the formation of "pre-tangle" tau pathology in the TG mice described earlier, we recently produced TG mouse lines that express 5-10 fold higher levels of wild type human 3R0N tau than endogenous mouse tau. These mice developed filamentous intraneuronal inclusions composed of tau and neurofilaments (Nfs), but the fibrils in these inclusions exhibited the ultrastructural features of straight filaments rather than PHFs characteristic of AD NFTs.

See Trojanowski at page 736, bottom left column (internal citations omitted). Trojanowski further states that "using model systems like those discussed here, it should be possible to make rapid progress in elucidating how and why glial and neuronal tau pathologies lead to the onset and progression of diverse sporadic and hereditary taupathies in the very near future." Thus, it cannot be concluded from Trojanowski that the production of transgenic animals that exhibit a specific phenotype would have required undue experimentation.

To support the Enablement rejection, the Examiner also stated that:

> the specification fails to provide any relevant teachings or sufficient guidance with regards to the production of any transgenic non-human animals comprising a transgenic sequence encoding SEQ ID NO: 1 or a sequence with 90% homology thereto, which over-expresses the transgenic sequence such that a phenotype occurs.

Paper No. 16, page 10, lines 16-19. This is an incorrect assessment of the teachings provided in the specification. The specification describes exemplary methods that may be

used to produce the transgenic animals of the invention. *See* Specification at page 20, lines 1-25. The specification also cites several references that describe various methods for producing transgenic animals. *See id.* In addition, methods for producing transgenic animals were well known in the art as of the effective filing date of the application. *See* Section VIII.C.2(a)(ii) (pages 23-24 of this Brief). It is unnecessary to supply information that is well known in the art. *See Genentech, Inc. v. Novo Nordisk*, 108 F.3d 1361, 1366, 42 USPQ2d 1001, 1005 (Fed. Cir. 1997). Thus, it is incorrect to assert that the specification fails to provide "any relevant teachings or sufficient guidance" regarding the production of the transgenic animals of the invention.

The Examiner also asserted that "the as-filed specification fails to describe any particular phenotype exhibited by any contemplated transgenic non-human animal of the invention when the nucleotide sequence is over-expressed in said animal." Paper No. 16, page 10, lines 19-22. This is an incorrect statement. Various phenotypes associated with the transgenic animals of the invention are described in the specification. For example, the specification at page 20, lines 26-29 indicates that:

> Once obtained, the transgenic animals which contain the AD7c-NTP may be analyzed by immunohistology for evidence of AD7c-NTP expression as well as for evidence of neuronal or neuritic abnormalities associated with Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas and glioblastomas.

In addition, in discussing the use of the transgenic animals of the invention for screening applications, the specification states that drug candidates can be tested by contacting them with a host (*e.g.*, a transgenic animal) transfected with a DNA construct comprising the DNA molecule of SEQ ID NO:1 or a DNA molecule that is at least 90% homologous thereto. *See* Specification at page 21, lines 7-10. Drug candidates can be identified, *e.g.*, on

the basis of their ability to reduce the frequency of at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons in the host. *See* Specification at page 21, lines 14-18. Thus, according to the specification, the transgenic animals of the invention may (but need not necessarily) exhibit neuronal or neuritic abnormalities associated with Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas and glioblastomas. More specifically, the phenotypes potentially exhibited by the transgenic animals of the invention, as set forth in the specification, include neuritic sprouting, nerve cell death, nerve cell degeneration, neurofibrillary tangles, and/or irregular swollen neurites. These phenotypes are supported by the results obtained when AD7c-NTP was over-expressed in cultured neuronal cells. *See* Specification at page 46, lines 1-26, and Figs. 6A-6G. Therefore, the assertion that the specification fails to describe "any particular phenotype" exhibited by the transgenic animals of the invention is incorrect.

The Examiner has also cited various factors that may, in general, influence the production of transgenic animals expressing a particular phenotype. *See* Paper No. 16, page 11, line 22, through page 12, line 6. The Examiner has cited four references that supposedly illustrate the difficulties associated with producing transgenic animals that exhibit a particular phenotype: Wall, R.J., *Theriogenology 45*:57-68 (1996) (copy attached hereto as Exhibit 16) (hereinafter "Wall"), Houdebine, L-M., *J. Biotechnology 34*:269-287 (1994), Mullins, L.J. and Mullins, J.J., *J. Clin. Invest. 97*:1557-1560 (1996) (copy attached hereto as Exhibit 17) (hereinafter "Mullins"), and Strojek, R.M. and Wagner, T.E., *Genetic Engineering: Principles and Methods 10*:221-246 (1988). *See* Paper No. 16, page 12. Based on these references, the Examiner concluded:

> it would require an undue amount of experimentation to reasonably predict the results achieved in any transgenic animal comprising a transgenic sequence set forth in SEQ ID NO:1 or a sequence with 90% homology thereto and which over-expresses the protein in the transgenic animal at the levels of the claimed product, the consequences of that production, and therefore, the resulting phenotype.

Paper No. 16, page 13, lines 8-12.

The references cited by the Examiner, rather than demonstrating that the production of transgenic animals with a particular phenotype requires undue experimentation, merely indicate that certain technical issues should be considered in order to successfully produce transgenic animals exhibiting a certain phenotype. To establish a *prima facie* case of non-enablement, it is not sufficient for an Examiner to show that *some* experimentation may be required to make and use the claimed invention; the Examiner must present evidence indicating that *undue* experimentation is required. *See Marzocchi*, 439 F.2d at 224, 169 USPQ at 370, *see also Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. The references cited by the Examiner simply describe certain challenges in the art. None of these references suggest that such challenges are insurmountable or that the production of transgenic animals with certain phenotypes would require undue experimentation.

In fact, the references cited by the Examiner actually support the position that producing transgenic animals with a particular phenotype *does not* involve a degree of experimentation that would be regarded as undue in the art. The cited references describe several instances in which transgenic animals exhibiting a desired phenotype were successfully produced. Wall, for example, summarizes the results of various researchers demonstrating the production of: (i) transgenic sheep with enhanced wool production characteristics, *see* Wall at page 59, third full paragraph; (ii) transgenic mice that serve as

models for human genetic diseases (including Alzheimer's disease), *see id.*; and (iii) transgenic pigs that express a human complement inhibitor for use in xenograft transplantation. *See id.* Mullins describes examples of the production of: (i) transgenic rats that successfully express the human apolipoprotein A-1 gene and that show increased serum HDL cholesterol concentrations, *see* Mullins at page 1558, right column, first full paragraph; (ii) transgenic rabbits expressing apoB-editing protein in the liver and exhibiting reduced LDL and lipoprotein(a) concentrations, *see id.*; (iii) transgenic rabbits expressing the human CD4 protein on T lymphocytes and exhibiting susceptibility to HIV infection, *see id.*; (iv) transgenic pigs expressing a bovine growth hormone gene and the resultant consequences on carcass tissue lipid composition, *see id.* at page 1558, paragraph bridging pages 1558-1559 (reference 23); and (v) transgenic swine expressing high levels of human hemoglobin, *see id.*

As described in Section VIII.C.2(c) (pages 26-29 of this Brief), there are many examples from the scientific literature that demonstrate the production of transgenic animals that exhibit particular phenotypes. The methods that were used to make such transgenic animals were available to persons skilled in the art at the time of the effective filing date of the application. The fact that there are numerous examples in the art of successfully produced transgenic animals expressing specific desired phenotypes, indicates that the production of such transgenic animals would not have require undue experimentation.

The Examiner has dismissed the evidence regarding the successful production of transgenic animals that exhibit particular phenotypes on the basis that "the exhibits do not use the same method and materials as contemplated by the specification." *See* Paper No. 16, page 15, lines 13-14, and Paper No. 18, page 2, lines 5-6. This is incorrect. It is clear from

the specification that any method known to those skilled in the art can be used for the production of the transgenic animals of the invention. *See* Section VIII.C.2(a)(ii) (pages 23-24 of this Brief). Thus, contrary to the Examiner's assertion, the methods used to produce the transgenic animals described, *inter alia*, in Exhibits 4-12, attached hereto, were contemplated by the specification.

Moreover, many of the references cited by Appellants describe the production of transgenic animals using *pronuclear microinjection*. The transgenic animals described in these references all exhibit particular phenotypes related to the transgene. Pronuclear microinjection is an exemplary method that is specifically discussed in the specification. *See* Specification at page 20, lines 3-17. It is therefore incorrect to state that these references "do not use the same method and materials as contemplated by the specification."

In summary, the methods that were used in Exhibits 4-12 to produce transgenic animals were contemplated by the specification and would have been available to persons having ordinary skill in the art at the time of the effective filing date of the application. It was therefore improper for the Examiner to not consider these references as evidence demonstrating the enablement provided for the present invention. In addition, the Examiner has failed to explain why it is believed that undue experimentation would have been required to produce the transgenic animals of Appellants' claims when numerous examples of the successful production of transgenic animals exhibiting specific phenotypes exist in the art.

With respect to the submitted examples demonstrating the successful production of transgenic animals that expressed amyloid beta genes or variants thereof and that exhibited neurological phenotypes indicative of Alzheimer's disease (Exhibits 8-12, attached hereto), the Examiner stated that "it is the specification, not the knowledge of one skilled in the art

that must supply the novel aspects of an invention in order to constitute adequate enablement." Paper No. 18, page 2, lines 13-15. The exhibits, however, were not submitted to demonstrate the novel aspects of the invention. Rather, the exhibits were submitted to demonstrate that transgenic animals exhibiting neurological phenotypes indicative of Alzheimer's disease had been successfully produced by others in the art. Accordingly, the exhibits directly refute the Examiner's position that producing transgenic animals with specific phenotypes would have required undue experimentation.

Finally, the Examiner referred to the following sentence from de la Monte and Wands, *J. Neuropathol. Exp. Neurol. 60*:195-207 (2001) (copy attached hereto as Exhibit 18), to support the enablement rejection:

> Although initial studies suggested that AD7c-NTP over-expression might contribute to AD neurodegeneration by promoting cell death, we were unable to investigate this issue using standard stably transfected cells because of progressive depletion of the cells in culture, which probably died due to apoptosis induced by AD7c-NTP expression.

de la Monte and Wands, sentence bridging pages 203-204, internal citation omitted, referred to in Paper No. 16, page 16, lines 11-16. Based on this sentence, the Examiner stated:

> If over-expression of AD7c-NTP resulted in cell death and depletion of cells in cultures, then one skilled in the art would conclude that overexpression of AD7c NTP in a transgenic non-human animal would result in cell death and depletion of cells in the animal. The specification does not teach one skilled in the art how to use the claimed transgenic non-human animal if overexpression of AD7c-NTP results in death of the transgenic animal.

Paper No. 18, page 2, lines 8-12.

If, as the Examiner suggests, overexpression of AD7c-NTP causes cell death in AD7c-NTP transgenic animals, it does not follow that making and/or using such transgenic

animals would require undue experimentation. The cells used in the experiments of de la Monte and Wands are PNET2 human CNS-derived neuronal cells. *See* de la Monte and Wands at page 196, left column. Since one of the phenotypes observed in the brains of patients with Alzheimer's disease is *neuronal* cell death, *see, e.g.*, Specification at page 2, lines 1-4, it is not surprising that over-expression of AD7c-NTP in *neuronal* cells resulted in cell depletion. There is no evidence to suggest that over-expression of AD7c-NTP in *non-neuronal* cells would result in cell depletion. Transgenic animals that over-express AD7c-NTP and that exhibit neuronal cell death would serve as useful models for AD and would be useful in screening applications to identify drugs to treat or prevent AD.

Moreover, the results of de la Monte and Wands do not necessarily indicate that over-expression of a DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto in one or more cells of the transgenic animals of the invention would necessarily result in death of the animals. Nonetheless, the possible death of the transgenic animals due to over-expression of SEQ ID NO:1 or a DNA molecule that is at least 90% homologous thereto does not indicate that producing the transgenic animals would have required undue experimentation or that the animals could not have been used in drug screening applications prior to the time of death. In addition, the phenotypes observed in transgenic animals postmortem would be instructive as to the physiological effects of AD7c-NTP in animal cells, and would provide a basis for developing treatments for diseases caused by an overabundance of AD7c-NTP.

Accordingly, Appellants respectfully request that the Board reverse the Examiner's § 112, first paragraph rejection of claims 7-9, 14-16, 35, 36 and 37-40 for alleged lack of enablement and remand this application for issue.

Respectfully submitted,

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IX. Appendix (37 C.F.R. § 1.192(c)(9))

7. A transgenic non-human animal, all of whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, wherein said DNA molecule is over-expressed in one or more cells of said transgenic animal, and wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when over-expressed in neuronal cells.

8. The transgenic non-human animal of claim 7, wherein the DNA molecule contained in each germ and somatic cell has SEQ ID NO:1.

9. The transgenic non-human animal of claim 7, wherein the protein coded for by said DNA molecule is overexpressed in the brain of the animal.

14. An *in vivo* method for screening a candidate drug that is potentially useful for the treatment or prevention of Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas, said method comprising:

(a) administering a candidate drug to the transgenic animal of claim 7, and

(b) detecting at least one of the following:

(i) the suppression or prevention of expression of the protein coded for by the DNA molecule contained by said animal; or

(ii) the increased degradation of the protein coded for by the DNA construct contained by said animal;

due to the drug candidate compared to a control animal which has not received the candidate drug.

15. The method of claim 14, wherein the DNA construct contained by said animal has SEQ ID NO:1.

16. The method of claim 14, wherein the protein coded for by the DNA construct contained by said animal is over-expressed in the brain of said animal.

35. The transgenic non-human animal of claim 7, wherein said transgenic animal is selected from the group consisting of non-human primate, mouse, sheep, pig, cattle, goat, guinea pig and rat.

36. The transgenic non-human animal of claim 7, wherein said activity of AD7c-NTP possessed by said DNA molecule when over-expressed in neuronal cells is selected from the group consisting of neuritic sprouting, nerve cell death, nerve cell degeneration, neurofibrillary tangles and irregular swollen neurites.

37. An *in vivo* method for screening a candidate drug that is potentially useful for the treatment or prevention of Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas, said method comprising:

(a) administering a candidate drug to the transgenic animal of claim 7, wherein said transgenic animal exhibits at least one of neuritic sprouting, nerve cell

death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons; and

(b) detecting the reduction of frequency of at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons in the host due to the drug candidate compared to a control animal which has not received the candidate drug.

38. The method of claim 37, wherein the DNA construct contained by said animal has SEQ ID NO:1.

39. The transgenic non-human animal of claim 7, wherein said transgenic animal is a vertebrate.

40. The transgenic non-human animal of claim 7, wherein said transgenic animal is a mammal.

Example 14: Product by Function

Specification: The specification exemplifies a protein isolated from liver that catalyzes the reaction of A $\longrightarrow$ B. The isolated protein was sequenced and was determined to have the sequence as set forth in SEQ ID NO: 3. The specification also contemplates but does not exemplify variants of the protein wherein the variant can have any or all of the following: substitutions, deletions, insertions and additions. The specification indicates that procedures for making proteins with substitutions, deletions, insertions and additions is routine in the art and provides an assay for detecting the catalytic activity of the protein.

Claim:

A protein having SEQ ID NO: 3 and variants thereof that are at least 95% identical to SEQ ID NO: 3 and catalyze the reaction of A $\longrightarrow$ B.

Analysis:

A review of the full content of the specification indicates that a protein having SEQ ID NO: 3 or variants having 95% identity to SEQ ID NO: 3 and having catalytic activity are essential to the operation of the claimed invention. The procedures for making variants of SEQ ID NO: 3 are conventional in the art and an assay is described which will identify other proteins having the claimed catalytic activity. Moreover, procedures for making variants of SEQ ID NO: 3 which have 95% identity to SEQ ID NO: 3 and retain its activity are conventional in the art.

A review of the claim indicates that variants of SEQ ID NO: 3 include but are not limited to those variants of SEQ ID NO: 3 with substitutions, deletions, insertions and additions; but all variants must possess the specified catalytic activity and must have at least 95% identity to the SEQ ID NO: 3. Additionally, the claim is drawn to a protein which **comprises** SEQ ID NO: 3 or a variant thereof that has 95% identity to SEQ ID NO: 3. In other words, the protein claimed may be larger than SEQ ID NO: 3 or its variant with 95% identity to SEQ ID NO: 3. It should be noted that "having" is open language, equivalent to "comprising".

The claim has two different generic embodiments, the first being a protein which comprises SEQ ID NO: 3 and the second being variants of SEQ ID NO: 3. There is a single species disclosed, that species being SEQ ID NO: 3.

A search of the prior art indicates that SEQ ID NO: 3 is novel and unobvious.

There is actual reduction to practice of the single disclosed species. The specification indicates that the genus of proteins that must be variants of SEQ ID NO: 3 does not have substantial variation since all of the variants must possess the specified catalytic activity and must have at least 95% identity to the reference sequence, SEQ ID NO: 3. The single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity. One of skill in the art would conclude that

applicant was in possession of the necessary common attributes possessed by the members of the genus.

Conclusion: The disclosure meets the requirements of 35 USC §112 first paragraph as providing adequate written description for the claimed invention.

e.g. expression vectors, the necessary common attribute is the ORF (SEQ ID NO: 2).

Weighing all factors including (1) that the full length ORF (SEQ ID NO: 2) is disclosed and (2) that any substantial variability within the genus arises due to addition of elements that are not part of the inventor's particular contribution, taken in view of the level of knowledge and skill in the art, one skilled in the art would recognize from the disclosure that the applicant was in possession of the genus of DNAs that comprise SEQ ID NO: 2.

Conclusion: The written description requirement is satisfied.

Example 9: Hybridization

Specification: The specification discloses a single cDNA (SEQ ID NO:1) which encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity. The specification includes an example wherein the complement of SEQ ID NO: 1 was used under highly stringent hybridization conditions (6XSSC and 65 degrees Celsius) for the isolation of nucleic acids that encode proteins that bind to dopamine receptor and stimulate adenylate cyclase activity. The hybridizing nucleic acids were not sequenced. They were expressed and several were shown to encode proteins that bind to a dopamine receptor and stimulate adenylate cyclase activity. These sequences may or may not be the same as SEQ ID NO: 1.

Claim:

An isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO: 1,

wherein said nucleic acid encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity.

Analysis:

A review of the full content of the specification indicates that the essential feature of the claimed invention is the isolated nucleic acid that hybridizes to SEQ ID NO: 1 under highly stringent conditions and encodes a protein with a specific function. The art indicates that hybridization techniques using a known DNA as a probe under highly stringent conditions were conventional in the art at the time of filing.

The claim is drawn to a genus of nucleic acids all of which must hybridize with SEQ ID NO: 1 and must encode a protein with a specific activity.

The search of the prior art indicates that SEQ ID NO: 1 is novel and unobvious.

There is a single species disclosed (a molecule consisting of SEQ ID NO: 1) that is within the scope of the claimed genus.

There is actual reduction to practice of the disclosed species.

Now turning to the genus analysis, a person of skill in the art would not expect substantial variation among species encompassed within the scope of the claims because the highly stringent hybridization conditions set forth in the claim yield structurally similar DNAs. Thus, a representative number of species is disclosed, since highly stringent hybridization conditions in combination with the coding function of DNA and the level of

skill and knowledge in the art are adequate to determine that applicant was in possession of the claimed invention.

Conclusion: The claimed invention is adequately described.

CREATING MANY MUTATIONS IN A DEFINED SEGMENT OF DNA

At present, it is impossible to predict with accuracy the effect of substituting one amino acid for another in a protein. Current attempts to "improve" the properties of a protein therefore depend on analyzing large numbers of variants that are created by site-directed mutagenesis in promising regions (e.g., in and around the active site of an enzyme). Clearly, the number of potential variations that can be created, even in a circumscribed region of a protein, is extremely large. For example, 114 different mutants would be required simply to insert every possible amino acid at each of six locations in a protein. This number grows to 6^{19} if such substitutions are made in a combinatorial fashion. When planning this type of mutagenesis, careful choices must therefore be made to keep the numbers of mutants within manageable limits. For example, the numbers of potential mutants can be markedly reduced by avoiding replacements that are (1) highly conservative (i.e., the substitution of one amino acid with another whose chemical properties are very similar), (2) highly radical (i.e., replacing an amino acid with another whose chemical properties are completely different), or (3) misguided (e.g., the substitution of cysteine residues in secretory proteins). However, when the number of desired mutants exceeds 20 or so, it becomes impractical and expensive to generate each of them individually using a separate mutagenic oligonucleotide. Methods have therefore been devised to use degenerate pools of oligonucleotides to create large populations of mutants in a single round of site-directed mutagenesis. These degenerate pools of oligonucleotides contain a mixture of normal and abnormal bases at each position in the sequence at which a mutagenic event is desired. In the remainder of this section, we present guidelines for ways in which these populations of clustered mutations can be efficiently generated using degenerate pools of mutagenic oligonucleotides.

Use of Degen rate Pools of Mutagenic Oligonucleotides

1. Pools of degenerate single-stranded oligonucleotides can be used only when the target amino acids are clustered. If all of the codons that are to be altered lie within a short stretch of contiguous nucleotides, a pool of degenerate single-stranded mutagenic oligonucleotides can be used as mismatched primers on single-stranded DNA templates to generate the corresponding set of mutants. However, the mutants cannot generally be distinguished from the original wild-type DNA by the standard method of screening by hybridization. In most cases, the mutagenic oligonucleotides are so long that there is no practical difference in stability between mismatched and perfect hybrids. Even if the oligonucleotides are sufficiently short (≤ 20 nucleotides in length), the pool usually contains many different members, each of which has different hybridization characteristics. It is therefore extremely difficult, if not impossible, to devise hybridization conditions that will distinguish all possible mutant sequences from the original wild-type sequences. This type of mutagenesis is therefore best carried out using the Kunkel system (see pages 15.74–15.79), which selects strongly against bacteriophages generated by replication of the original wild-type (+) strand of DNA. Mutants are then identified by picking individual plaques blindly and sequencing the relevant section of single-stranded bacteriophage DNA.

2. An alternative method is to generate pools of mutants by "cassette mutagenesis," a technique that involves replacing the wild-type sequence with synthetic double-stranded oligonucleotides (see, e.g., McNeil and Smith 1985; Wells et al. 1985; Derbyshire et al. 1986; Hill et al. 1986, 1987; Hutchison et al. 1986; Bedwell et al. 1989). Since cassette mutagenesis was first introduced (Matteucci and Heyneker 1983), several variations have been described, each of which has advantages under particular circumstances. However, all of these techniques suffer from a common drawback—the necessity for unique restriction sites at both ends of the cassette. Because these restriction sites are required to shuttle the synthetic double-stranded oligonucleotide into the correct location, they cannot occur anywhere else in either the plasmid vector or the segment of the wild-type gene that it carries. Furthermore, to ensure that the cassette is inserted in the correct orientation, the cassette should carry different restriction sites at each end. Because naturally occurring restriction sites hardly ever fulfill these criteria, it is usually necessary to carry out one or more rounds of site-directed mutagenesis to create suitable restriction sites at the appropriate locations in the wild-type gene. If the introduction of these sites changes the amino acid sequence encoded by the gene, it is necessary to determine whether the resulting protein displays wild-type characteristics. To eliminate the possibility that the phenotypes of any mutants obtained by cassette mutagenesis result from a combination of amino acid changes (i.e., changes caused by introduction of the restriction sites and by changes encoded within the cassette), it may be necessary to restore the original wild-type sequence at the restriction sites.

   Three different methods are currently used to generate double-stranded

oligonucleotide cassettes. In the first method (McNeil and Smith 1985) (see Figure 15.9A), two separate sets of oligonucleotides are synthesized that are complementary to the opposite strands of the target DNA. One of these sets consists of a single species of oligonucleotide that is exactly complementary to the sequence of one of the strands of the wild-type target DNA. The other set consists of a degenerate pool of oligonucleotides that is complementary to the opposite strand and that carries the desired mutations. These sets of complementary oligonucleotides are then mixed under conditions that will allow mismatched hybrids to form. If the complementary oligonucleotides have been designed to yield double-stranded cassettes that carry the appropriate protruding termini, they can be inserted directly into a recombinant plasmid in place of the homologous wild-type sequence. Alternatively, cohesive termini can be created by digesting double-stranded blunt-ended cassettes with the appropriate restriction enzymes. The mismatches in the recombinant plasmids are repaired in vivo, after the recombinant plasmids have been introduced into competent bacteria. Subsequent replication of the plasmid DNA and segregation into daughter cells allows clones to be isolated that are derived from each DNA strand of the plasmid originally used for transformation. In this method and the one that follows, the plasmids isolated from individual colonies of transformed bacteria are occasionally heterogeneous, suggesting that segregation of the plasmids is sometimes incomplete. This problem can be solved by retransforming competent bacteria with plasmid DNAs extracted from pooled primary transformants. However, in this first method of cassette mutagenesis, the frequency of mutation can never exceed 50% because only one half of the progeny plasmids are derived from the mutagenized strand.

In the second method (see Figure 15.9B), the frequency of mutation is increased by using cassettes in which the complementary strands both consist of mixed-sequence oligonucleotides (Wells et al. 1985). Because each of these strands gives rise to progeny plasmids, the mutation rate can be raised to greater than 50% (Makris et al. 1988).

In the third method (see Figure 15.9C), degenerate pools of single-stranded oligonucleotides are converted to a blunt-ended double-stranded form by mutually primed synthesis (Oliphant et al. 1986; Hill et al. 1987). Two degenerate pools of oligonucleotides are synthesized that are complementary to the same strand of the target DNA. However, the members of one pool carry sequences at their 3′ termini that are complementary to sequences at the 3′ termini of oligonucleotides in the second pool. Usually, these complementary sequences are palindromic and correspond to the restriction site that marks one end of the cassette. The oligonucleotides in the two pools are then annealed to form partial hybrids that can be converted to blunt-ended double-stranded DNA by the Klenow fragment of *E. coli* DNA polymerase I. The products of this reaction are tail-to-tail dimers. Unit-length cassettes are generated by digesting the dimers with the appropriate restriction enzymes.

The major advantage of the third method is that the unit-length cassette consists of perfect homoduplexes. Any potential bias that occurs during mismatch repair in vivo is therefore avoided, and there is no loss of

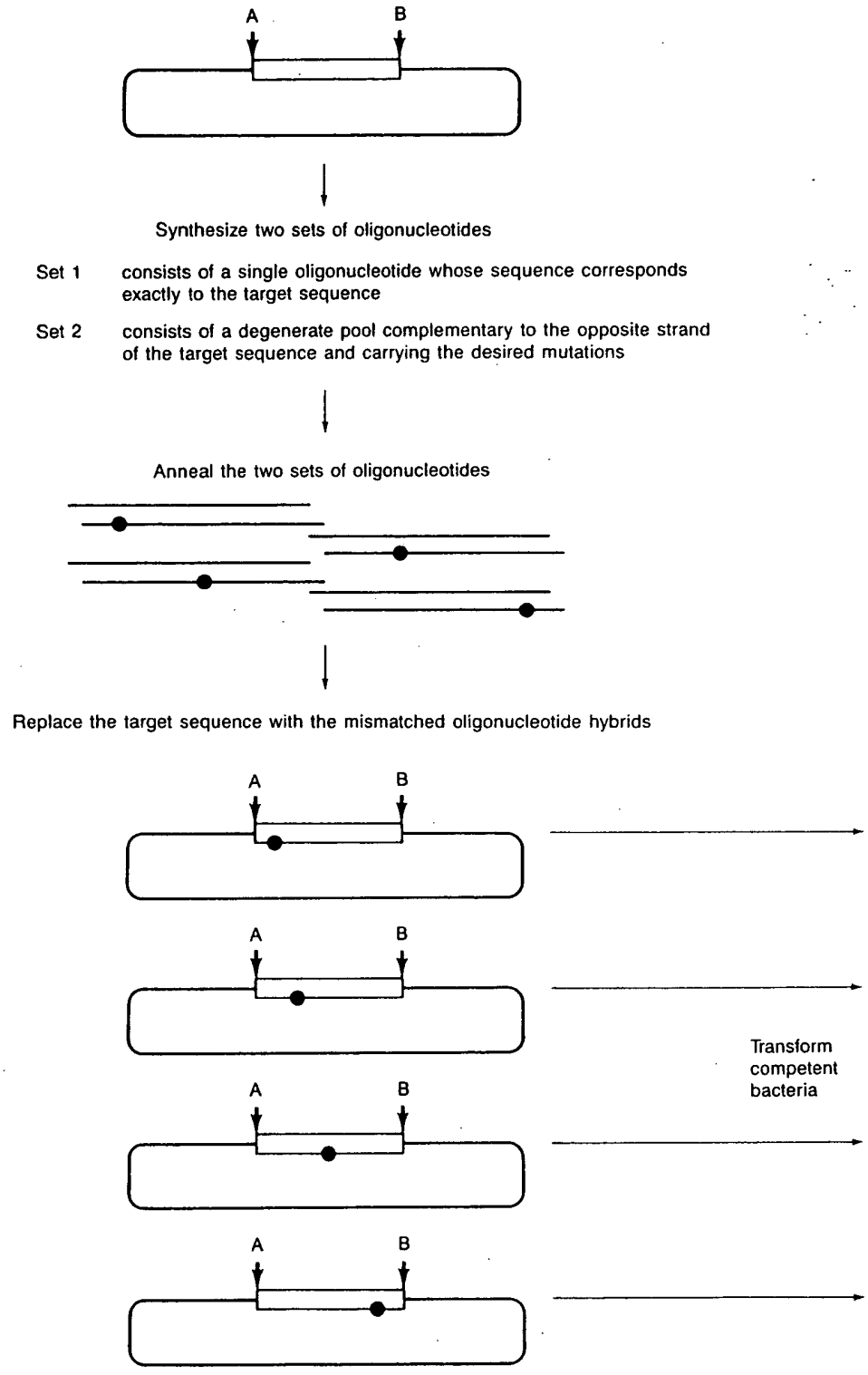
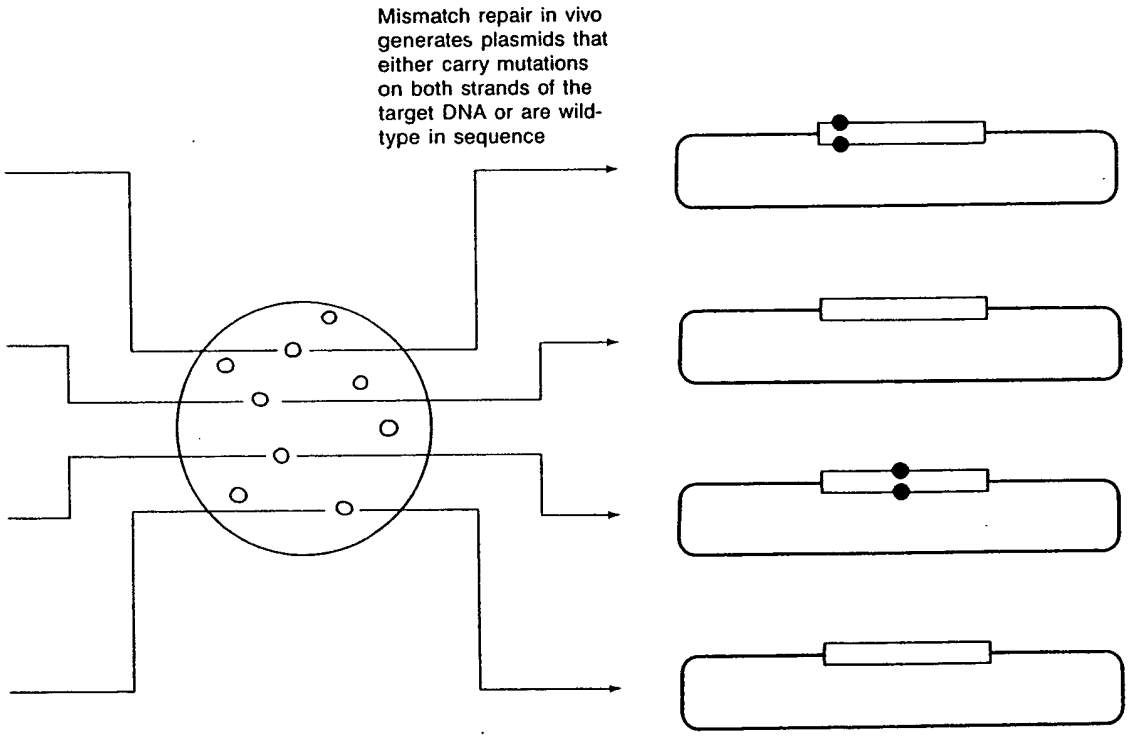


FIGURE 15.9A
Cassette mutagenesis using a single mixed-sequence oligonucleotide and repair of mismatches in vivo.


Mismatch repair in vivo generates plasmids that either carry mutations on both strands of the target DNA or are wild-type in sequence

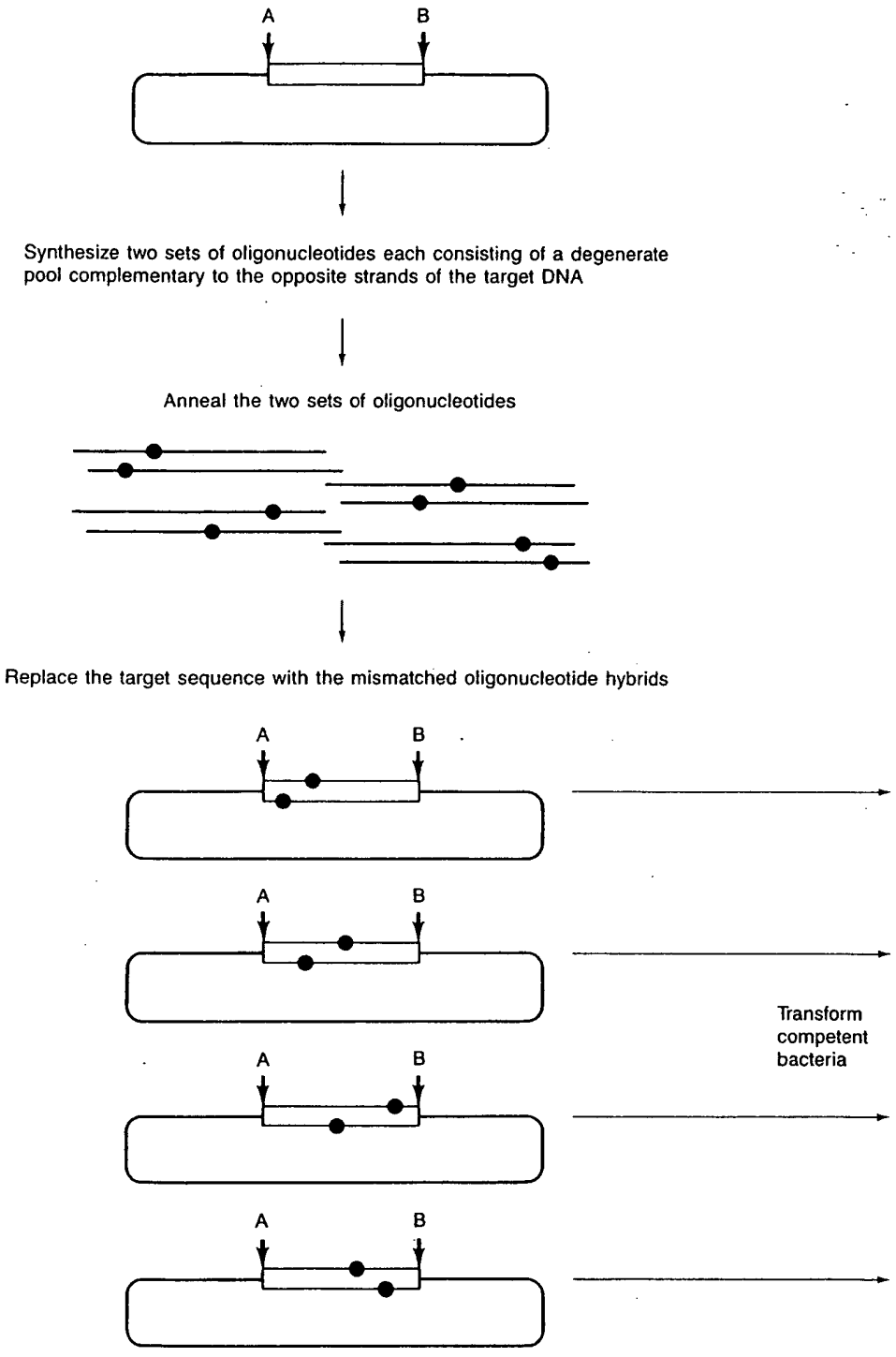
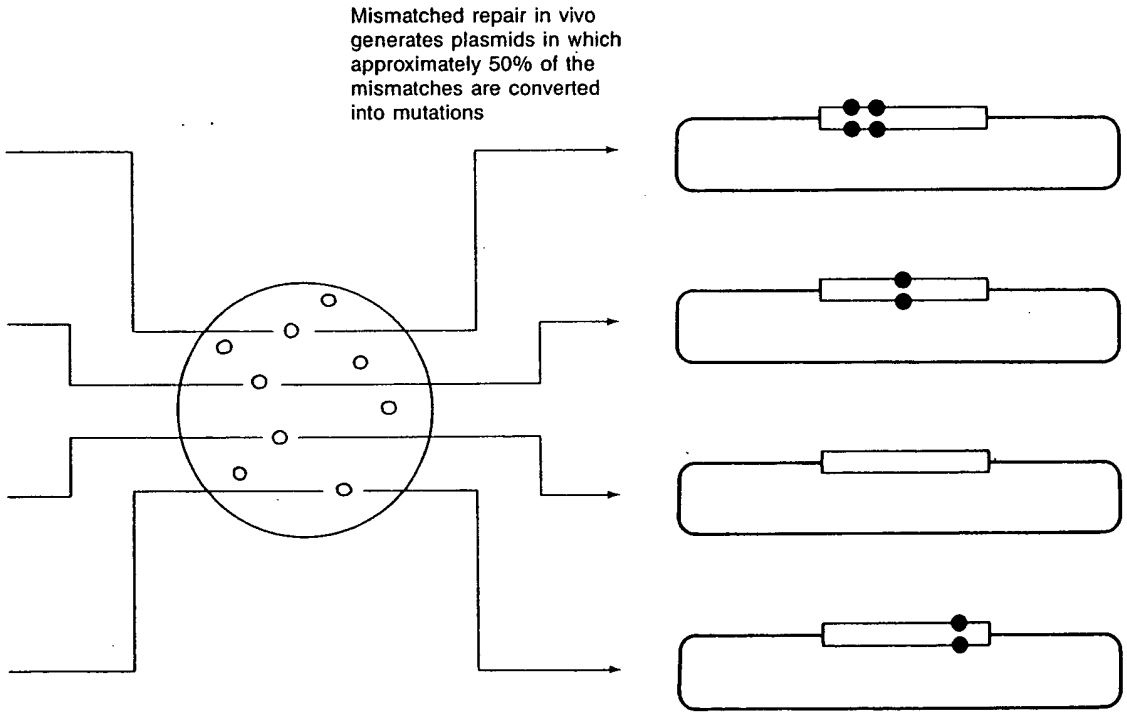


FIGURE 15.9B
Cassette mutagenesis using two complementary mixed-sequence oligonucleotides and repair of mismatches in vivo.


Mismatched repair in vivo generates plasmids in which approximately 50% of the mismatches are converted into mutations

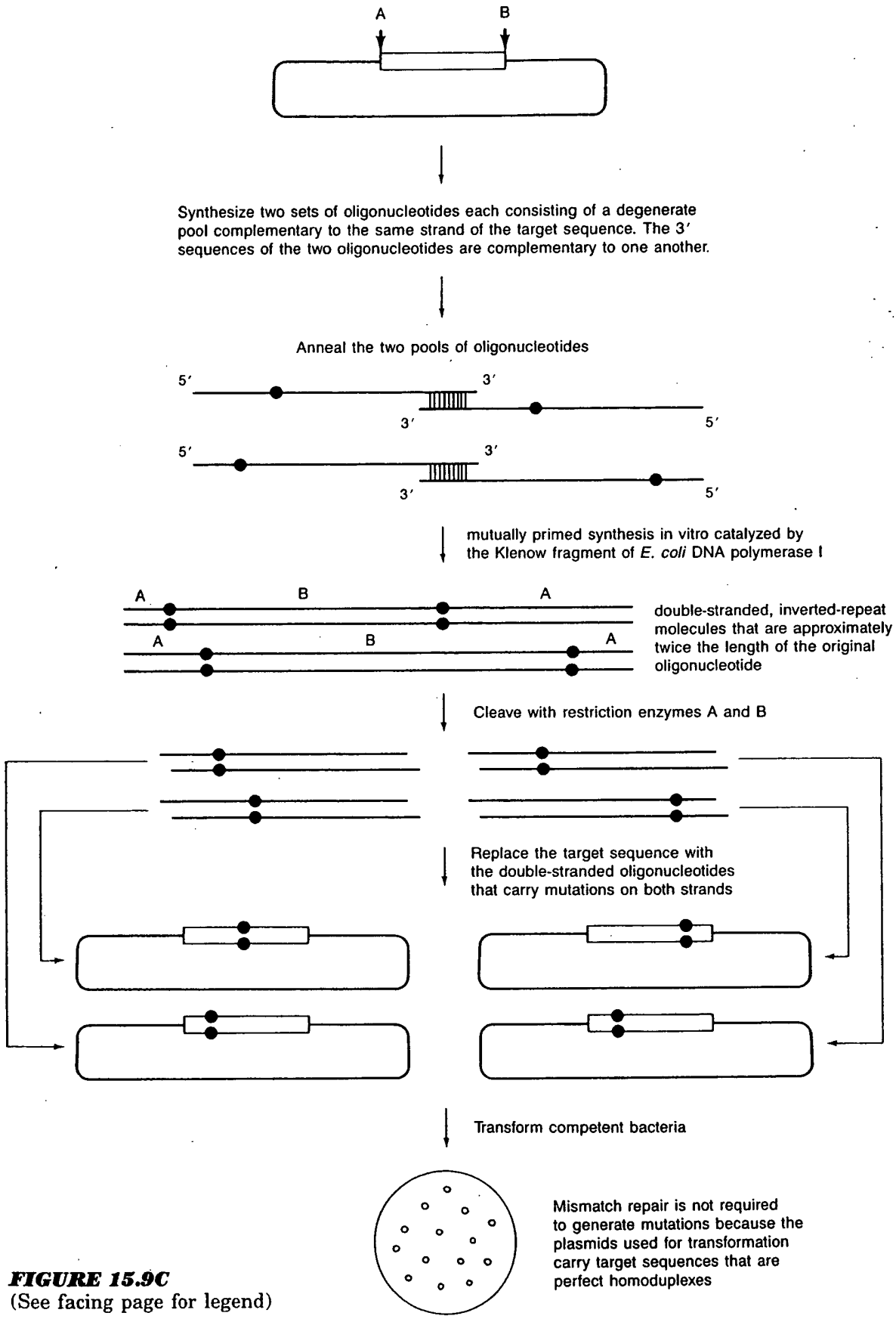


FIGURE 15.9C
(See facing page for legend)

mutants because of correction to wild-type sequences. Finally, because segregation is not required, primary bacterial transformants contain pure plasmid populations that can be analyzed directly. For these reasons, this is currently the method of choice for creating mutations at many sites within a defined region of DNA.

3. Ideally, each member of a degenerate pool of oligonucleotides should contain one nucleotide change per target sequence. In practice, however, because the oligonucleotide pools are generated in a mixed synthetic reaction, the best that can be achieved is an *average* of one altered base per target sequence. At each cycle, therefore, there is a chance that either a normal or an altered base will be incorporated into a growing oligonucleotide chain. The mutation frequency at any given site depends on the relative concentrations of the different nucleotide precursors that are provided at a particular cycle in the synthetic reaction.

   The fraction of oligonucleotides that contain nucleotide changes follows a binomial distribution that can be predicted from the following equation (McNeil and Smith 1985; Makris et al. 1988):

   $$F(P) = n!P^{n-r}(1-P)^r/(n-r)!r!$$

   where $F(P)$ is the fraction of the population whose sequence contains r random base changes over a target sequence of n consecutive bases, P is the probability of any given nucleotide being unchanged, and $(1-P)$ is the probability of any given nucleotide being changed. For example, when the length of the target sequence is 20 and the mixture of precursors supplied at every cycle contains 95% of the "normal" nucleotides and 5% of the "altered" nucleotides, the fraction of oligonucleotides that contain one altered nucleotide ($r = 1$) can be calculated as follows:

   $$F(P) = 20!(0.95)^{19}(0.05)^1/19!1!$$
   $$= 0.38$$

   Similarly, 36% of the oligonucleotides in the pool will contain no alteration in nucleotide sequence; 19% of the oligonucleotides will contain two changes, and 7% will contain more than two changes.

4. The types of mutations created by degenerate pools of oligonucleotides depend on the precursors that are supplied at each round of the synthetic cycle. In the example discussed above, not more than 5% of the precursors provided at each round of synthesis can contain "abnormal" nucleotides. Within this 5%, however, the ratio of the three abnormal bases can be altered according to the needs of the particular experiment. Because transversions are usually more useful than transitions, many workers increase the proportion of abnormal bases that will generate transversions at the expense of abnormal bases that will cause transitions.

5. The termini of the oligonucleotides should not be mutagenized because they will be needed to insert the cassette into the appropriate plasmid. If

FIGURE 15.9C
Cassette mutagenesis using two partially overlapping mixed-sequence oligonucleotides and complementary strand synthesis in vitro.

cohesive termini are to be generated by cleaving the double-stranded cassettes with restriction enzymes, three extra nucleotides should be added to each end of the mutagenic oligonucleotide. These extensions increase the efficiency of digestion with restriction enzymes.

6. The frequency with which mutants are obtained at any particular position decreases as the length of the mutagenic oligonucleotides in the degenerate pool increases. Because individual mutants are recovered by random sampling, it is improbable that all possible mutations will be isolated when the size of the potential pool is large. Under these circumstances, "missing" clones that carry particularly interesting mutations can usually be identified by hybridization to specifically designed oligonucleotide probes.

Finally, it is worth remembering that oligonucleotide-mediated mutagenesis is not the only method that can be used to saturate segments of cloned DNA with mutations. Several of the other techniques that are available are discussed below.

Treatment of Double-stranded DNA with Chemical Mutagens

The simplest method of localized random mutagenesis is to react a short fragment of double-stranded DNA with a chemical mutagen such as nitrous acid or hydroxylamine and to clone the population of mutagenized fragments into a recombinant plasmid that carries the remainder of the wild-type gene. Recombinant plasmids carrying mutations that generate a novel phenotype can be recognized by appropriate tests. For example, a temperature-sensitive mutation constructed in a gene coding for a mammalian protein might be recognized by immunofluorescent staining of mammalian cells that had been transfected with the appropriate plasmid incubated at permissive and non-permissive temperatures. Recombinant plasmids carrying a mutation that does not give rise to an easily assayed phenotype must be identified by DNA sequencing of random clones. Unfortunately, the frequency at which mutants are recovered by this method is unacceptedly low (Chu et al. 1979; Solnick 1981; Busby et al. 1982; Kadonaga and Knowles 1985). Furthermore, because chemical mutagens react with bases in double-stranded DNA in a highly specific manner, only a limited spectrum of mutations is recovered. For these reasons, this method is no longer in widespread use.

Treatment of Single-stranded DNA with Sodium Bisulfite

In the original descriptions of this protocol, circular double-stranded plasmid DNA was nicked at a random site with pancreatic DNAase I in the presence of ethidium bromide (Greenfield et al. 1975; Shortle and Botstein 1983). The nick was then converted to a gap by digestion with exonuclease III, and the resulting gapped double-stranded molecule was exposed at slightly acid pH to sodium bisulfite (1–3 M), which caused deamination of cytosine to uracil. After transformation of bacteria, replication of the mutagenized DNA led to replacement of the original C:G base pair with a T:A base pair. Recently, the efficiency of this type of mutagenesis has been improved by carrying out deamination on gapped duplexes of bacteriophage M13 recombinants in which the target DNA is exposed in a single-stranded form (Pine and Huang 1987). After mutagenesis, the DNA is transfected into an *ung*$^-$ strain of *E. coli* that is unable to remove the newly generated uracil residues. Although the procedure results in highly efficient mutagenesis of a defined segment of DNA, it generates only transition mutations in which a purine replaces a purine on one strand of DNA and a pyrimidine replaces a pyrimidine on the other. Unfortunately, mutations of this type generally result in conservative substitutions of amino acids. Thus, the range of mutants that are obtained is often too narrow to allow a comprehensive analysis of a particular segment of a protein (Shortle and Nathans 1978; DiMaio and Nathans 1980; Peden and Nathans 1982).

Treatment of Single-stranded DNA with Chemicals That Damage All Four Bases

In this method (Myers et al. 1985a), single-stranded DNA of a recombinant M13 bacteriophage is exposed under defined conditions to chemicals (nitrous acid, formic acid, and hydrazine) that modify bases in single-stranded DNA without breaking the phosphodiester backbone (see Chapter 13). After removal of the chemicals, a universal sequencing primer and avian reverse transcriptase are used to synthesize the complementary strand of DNA. When the polymerase encounters damaged bases in the template strand, it incorporates nucleotides essentially at random. Because all possible nucleotides can be incorporated at a single position, there is a 75% probability of mutation at every site of damage. Furthermore, because tranversions are generated twice as frequently as transitions, the resulting mutations generate proteins with a wide spectrum of amino acid changes. After the extension reaction is completed, the double-stranded target fragment is excised and recloned into an appropriate vector. Mutants can be identified directly by DNA sequencing of random clones.

The major problem with this method is the frequency with which useful mutations can be isolated. To prevent the formation of unacceptable numbers of multiple mutants, it is necessary to limit carefully the length of time the single-stranded DNA is exposed to damaging chemicals. However, this means that many of the template strands escape modification altogether. Therefore, the best that can be achieved by this method is a frequency of single mutations of 10–15%. This problem can sometimes be alleviated by using denaturing gradient gel electrophoresis to purify fragments of DNA that carry mutations (Myers et al. 1985a,b). However, this technique is by no means simple, and it requires the attachment of the mutagenized DNA to special vectors equipped with GC clamps (Myers et al. 1985c). Because of these problems, this method of mutagenesis has so far not found widespread acceptance.

Misincorporation of Nucleotides by DNA Polymerases

Point mutations can be introduced into double-stranded DNA by incorporating base analogs with various types of DNA polymerases. For example, Shortle and his coworkers (Shortle et al. 1982; Shortle and Lin 1985) incubated gapped DNA in the presence of *E. coli* DNA polymerase I and only one of the four α-thiophosphate dNTPs. Thiophosphate dNTPs are efficiently incorporated by the polymerase but are not effectively removed by its $3' \rightarrow 5'$ editing function. The incorrect base is thus incorporated at a high frequency, and the remainder of the gap is then filled in a second polymerization reaction carried out in the presence of all four of the normal dNTPs. All types of base substitutions have been obtained with this method using each of the four α-thiophosphate dNTPs in separate repair reactions.

Another misincorporation method uses AMV reverse transcriptase, which is deficient in a $3' \rightarrow 5'$ exonuclease activity (Zakour and Loeb 1982). In this case, conventional dNTPs are used to synthesize DNA from an upstream primer. Base analogs are then incorporated in the region of interest.

The major problem of these and other misincorporation methods is the difficulty in creating populations of template molecules in which the 3′ hydroxyl terminus of the growing strand is located at random positions throughout the region of interest. Although this can in theory be achieved by a number of different methods (e.g., controlled nick translation with *E. coli* DNA polymerase I or controlled digestion of double-stranded DNA with exonuclease III), the routine generation of large numbers of mutations at random sites has proved to be difficult in practice. Success requires careful characterization of the reagents involved, meticulous establishment of optimal reaction conditions, and many trial experiments.

In summary, whereas methods (discussed earlier in this chapter) to introduce single mutations in cloned DNA are now well-established, techniques to saturate defined regions with mutations are less satisfactory. Using chemical mutagenesis, the rate of production of single mutations is low and/or the mutations themselves are of limited interest. Using misincorporation of base analogs, it is difficult to direct the mutations to the region of interest. However, it seems likely that at least some of these problems will be solved during the next few years, for example, by incorporating base changes into DNA synthesized in polymerase chain reactions or by advances in DNA chemistry that will facilitate the synthesis of mutagenized DNAs of extended length. Until then, we recommend using degenerate pools of synthetic oligonucleotides. In contrast to the other methods, the mutations can be precisely designed by the experimenter and can be focused in a defined region of DNA. Even with these limitations, the amount of work involved in isolating and characterizing a comprehensive set of mutants remains very large. In this branch of molecular cloning, therefore, it is especially important to weigh the potential scientific rewards against the commitment of time and personnel that the project will certainly consume.

Cell, Vol. 63, 1099–1112, December 30, 1990, Copyright © 1990 by Cell Press

Spontaneous Inflammatory Disease in Transgenic Rats Expressing HLA-B27 and Human β_2m: An Animal Model of HLA-B27-Associated Human Disorders

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Summary

Humans who have inherited the human class I major histocompatibility allele HLA-B27 have a markedly increased risk of developing the multi-organ system diseases termed spondyloarthropathies. To investigate the role of B27 in these disorders, we introduced the B27 and human β_2-microglobulin genes into rats, a species known to be quite susceptible to experimentally induced inflammatory disease. Rats from one transgenic line spontaneously developed inflammatory disease involving the gastrointestinal tract, peripheral and vertebral joints, male genital tract, skin, nails, and heart. This pattern of organ system involvement showed a striking resemblance to the B27-associated human disorders. These results establish that B27 plays a central role in the pathogenesis of the multi-organ system processes of the spondyloarthropathies. Elucidation of the role of B27 should be facilitated by this transgenic model.

Introduction

Class I major histocompatibility (MHC) gene products are polymorphic 44,000 M_r glycoproteins expressed on cell surfaces in noncovalent association with the nonpolymorphic 12,000 M_r light chain β_2-microglobulin (Klein, 1986). Among class I MHC molecules, HLA-B27, a serologically defined allele of the human *HLA-B* locus, is of particular interest because it is uniquely associated with a group of relatively common inflammatory disorders. The strongest association is seen with primary ankylosing spondylitis, a chronic inflammatory disease affecting the axial musculoskeletal system: ~90% of affected individuals have inherited the B27 allele in comparison with only ~7% of Caucasians in the general population (Brewerton et al., 1973; Schlosstein et al., 1973; Tiwari and Terasaki, 1985). An important association also exists between HLA-B27 and reactive arthritis, in which certain microbial infections of the gastrointestinal or genitourinary tracts trigger inflammation in joints and other tissues (Toivanen and Toivanen, 1988). A summary of the major disorders associated with HLA-B27 is presented in Table 1.

The B27-associated diseases are classified as rheumatic disorders because of the prominence of musculoskeletal manifestations. Nonetheless, all of these diseases can involve multiple organ systems, particularly the gastrointestinal tract, genitourinary tract, skin, eye, and heart. Because of the overlap among these diseases with regard to epidemiology, clinical manifestations, and anatomic pathology, they were recognized as a distinct cluster of interrelated diseases, termed spondyloarthropathies, even before the common genetic marker of B27 was identified (Moll et al., 1974). Thus it has long been speculated that a common pathogenetic mechanism might underlie the association of B27 with this heterogeneous group of disorders. Despite extensive investigation, however, the etiology and pathogenesis of these diseases have remained obscure, and the basis for the association with B27 has not been established.

In an attempt to develop an animal model of B27-associated disease, we (Taurog et al., 1988a) and others (Krimpenfort et al., 1987; Nickerson et al., 1990; Weiss et al., 1990) have produced transgenic mice expressing HLA-B27 and human β_2-microglobulin (hβ_2m). However, despite physiologically normal function of B27 in both hybrid and inbred mice (Kievits et al., 1987; Taurog et al., 1988a) and a reported influence of B27 on the course of an experimental bacterial infection in mice (Nickerson et al., 1990), no faithful reproduction of any of the features of B27-associated human disease has been reported in transgenic mice. These negative results raised the possibility that susceptibility to the spondyloarthropathies might not be related to the B27 gene. Alternatively, other features of the mouse may not have permitted expression of the relevant pathologic changes. We therefore sought to develop transgenic technology in rats, which are susceptible to several experimentally induced arthritic diseases that cannot be elicited in mice (Greenwald and Diamond, 1988).

In this paper, we describe the production of transgenic rats that express HLA-B27 and hβ_2m genes. We further describe a disorder spontaneously arising in these B27 transgenic rats that includes most of the features of B27-associated disease in humans.

Results

Integration of HLA-B27 and hβ_2m Genes in Inbred Rats

Fertilized one-cell rat eggs were microinjected with a solution containing both DNA fragments shown in Figure 1. The HLA-B27 gene encoding the HLA-B*2705 subtype was contained on a 6.5 kb EcoRI fragment that included 0.7 kb of 5′ flanking sequence and 2.5 kb of 3′ flanking sequence (Figure 1A). The hβ_2m gene was contained on a 15 kb SalI–PvuI fragment that included 5.2 kb of 5′ flanking sequence and 1.9 kb of 3′ flanking sequence (Figure 1B). Identification and quantitation of transgenes in the founder animals and their progeny were determined by dot-blot hybridization of genomic DNA isolated from tail bi-

Table 1. Rheumatic Diseases Associated with HLA-B27

Characteristic	Disorder				
	Ankylosing Spondylitis	Reactive Arthritis[a]	Juvenile Spondyloarthropathy	Psoriatic Arthropathy	Enteropathic Arthropathy
Sacroiliitis or spondylitis[b]	100%	<50%	<50%	20%	10%
Peripheral arthritis[c]	25%	90%	90%	95%	90%
Gastrointestinal inflammation	Common, usually asymptomatic	Common, often symptomatic	Not known	Uncommon	All
Skin and nail involvement	Rare	Most	Uncommon	All	Uncommon
Genitourinary involvement (males only)	Uncommon	Most	Uncommon	Uncommon	Rare
Eye involvement[d]	25%	Common	Common	Occasional	Occasional
Cardiac involvement	<5%	5%–10%	Not known, probably rare	Rare	Rare
Usual age of onset (years)	18–40	18–45	7–18	20–50	15–50
Sex prevalence	Males 3:1	Males 3:1[e]	Males 10:1	Equal	Equal
Type of onset	Gradual	Acute	Variable	Variable	Gradual
Role of infectious agents	Unknown	Definite Trigger	Unknown	Unknown	Unknown
Prevalence of HLA-B27[f]	>90%	60%–80%	80%	50%[g]	50%–75%[g]

Table adapted from Calin (1984); Tiwari and Terasaki (1985); Khan and van der Linden (1990); Taurog and Lipsky (1990).
[a] Includes Reiter's syndrome, classically defined as the triad of arthritis, conjunctivitis, and urethritis.
[b] Inflammation in the spine or sacroiliac joints.
[c] Inflammation in joints of the extremities.
[d] Predominantly conjunctivitis in reactive arthritis; iritis with the other disorders.
[e] Male to female ratio is 10:1 if venereally acquired; 1:1 if enteropathically acquired.
[f] Caucasians of northern European extraction only. General prevalence in this population is 6%–8%. Some variation seen in other populations, but the basic associations with HLA-B27 are seen worldwide.
[g] Frequency elevated only in those with spondylitis or sacroiliitis.

opsies. Hybridization was carried out with 5′ and 3′ flanking probes for the HLA-B27 gene (probes A and C in Figure 1A), and with a 3.7 kb BglII fragment containing exons 2 and 3 of the $h\beta_2m$ gene (probe D in Figure 1B).

Seven LEW and four F344 rats that developed from microinjected ova showed integration of the HLA-B27 and $h\beta_2m$ genes. Of these, four LEW rats and one F344 rat showed cell surface expression of both HLA-B27 and $h\beta_2m$, as assessed by indirect immunofluorescence of peripheral blood lymphocytes (PBLs). One additional LEW rat showed integration and expression of the B27 gene alone. Table 2 summarizes the results of the microinjection experiments.

All of the founder rats expressing the transgenes were subsequently shown to transmit the transgenes to their offspring. One of the six founders, 21-3, was found to be a mosaic, based on non-Mendelian rates of transmission and on enhanced cell surface expression in the offspring. Another founder, 21-4, a female, was shown to have two independently segregating loci of transgene integration, each locus carrying both transgenes. One line arising from this found r, inheriting a locus containing 150 copies of th B27 gen and 90 copies of th $h\beta_2m$ gene, was t rmed 21-4H. Th other line, inheriting a locus containing six copies of the B27 gene and six copies of the $h\beta_2m$ gene, was termed 21-4L (Table 3).

Lymphocyte Cell Surface Expression of the HLA-B27 and $h\beta_2m$ Transgene Products

Expression of the transgene products was estimated by indirect immunofluorescence and flow cytometry of PBLs stained with specific monoclonal antibodies. The relative expression of B27 and $h\beta_2m$ in seven transgenic lines is shown in Table 3. To compensate for interexperiment variation, the mean channel fluorescence for each line with each antibody is expressed relative to that determined in the same experiment for PBLs of the transgenic mouse line 56-3, which expresses high levels of both B27 and $h\beta_2m$ on PBL surfaces. The highest expression of both gene products was found in the LEW lines 21-4H and 21-4L and the F344 line 33-3.

The patterns of cell surface expression of B27 and $h\beta_2m$ in the 21-4H and 21-4L lines are shown in Figures 2A and 2B. The binding of the endogenous rat class MHC I molecules (RT1) to the anti-RT1 antibody OX18 is shown in Figur 2C for both transgenic lines and the nontransgenic control. Th levels of expression of B27 and $h\beta_2m$ wer comparable in th two transgenic lin s (Figures 2A

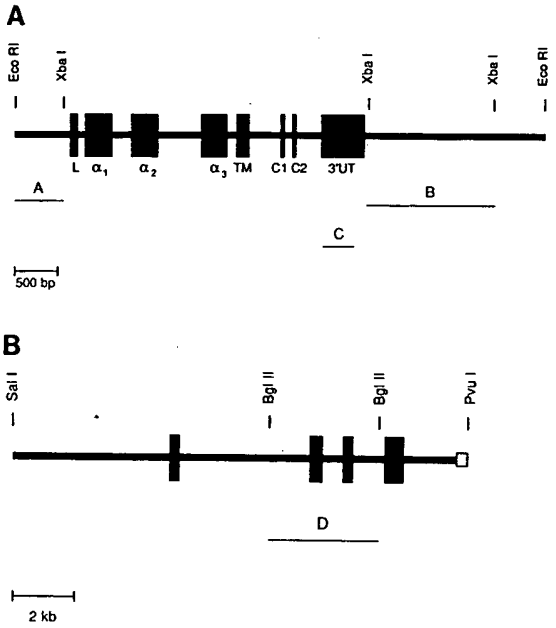

Figure 1. Genes Used for Microinjection of Fertilized Rat Eggs

(A) The HLA-B* 2705 gene (clone pE.1-B27) was contained on a 6.5 kb EcoRI fragment. Exons are indicated by boxes and labeled. Probes from the 5′ and 3′ flanking regions, labeled A and B, respectively, were used for dot-blot hybridization of genomic DNA. Probe C, from the 3′ untranslated region, was used for Northern hybridization.

(B) The hβ2m gene (clone pβ2m-13) was contained on a 15 kb SalI–PvuI fragment. Exons are indicated by boxes. The insert contained ~100 bp of the vector pEMBL9, indicated by the open box at the 3′ end. The 3.7 kb BglII fragment labeled D was used for both dot-blot hybridization of genomic DNA and for Northern hybridization.

Table 2. Production of HLA-B27 and hβ2m Transgenic Rats

			Founder			
			Integration[b]		Expression[c]	
Strain	Eggs[a]	Pups	B27	hβ2m	B27	hβ2m
LEW	348	23	8	7	5	4
F344	329	24	4	4	1	1

[a] Number of eggs injected and transferred to pseudopregnant recipients.

[b] Transgenic animals were identified by dot-blot analysis of DNA isolated from tails.

[c] Cell surface expression was assessed by indirect immunofluorescence and flow cytometry of PBLs.

and 2B), and in both lines the expression of the endogenous RT1 class I molecules appeared to be reduced in comparison with the nontransgenic control (Figure 2C).

Immunologic Function of the HLA-B27 Transgene

To assess T cell recognition of the B27 transgene product as a class I MHC antig n, primary grafts of B27 transgenic LEW rat skin were placed on nontransgenic LEW rats, and spleen cells from the r cipient rats w re subsequently test d for B27-specific cytotoxicity. As shown in Table 4,

Table 3. Copy Number and Cell Surface Expression of HLA-B27 and hβ2m in Transgenic Rat Lines

	Gene (Copy/Cell)[a]		Cell Surface Expression (Relative MCF)[b]	
Line	B27	hβ2m	B27	hβ2m
21-2	1	1	0.09	0.06
21-3	20	15	0.30	0.29
21-4L	6	6	0.74	0.42
21-4H	150	90	0.51	0.42
25-1	1	0	0.15	0.00
25-6	7	7	0.42	0.27
33-3	55	66	1.00	0.76

[a] Gene copy number was estimated by quantitative dot hybridization on DNA isolated from tails using probes specific for each transgene (see Figure 1A).

[b] Mean channel fluorescence (MCF) with antibodies to HLA-B (B1.23.2) or hβ2m (BBM.1) of PBLs from transgenic rats, relative to simultaneously determined MCF of PBLs from the B27/hβ2m transgenic mouse line 56-3. All data are from progeny of founders to eliminate influence of mosaicism.

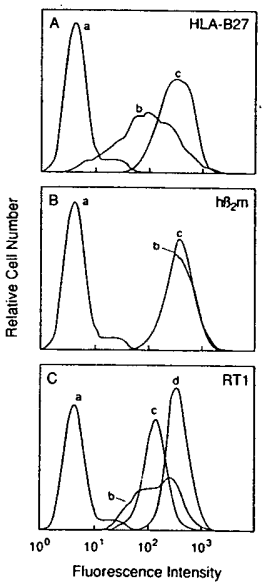

Figure 2. Comparison of Cell Surface Expression of HLA-B27, hβ2m, and the Endogenous RT1 Class I MHC Molecules in 21-4H, 21-4L, and Nontransgenic Rats

Peripheral blood mononuclear cells were incubated with saturating concentrations of monoclonal antibodies and fluorescein-labeled second antibodies and then analyzed by flow cytometry, as described in Experimental Procedures. The results demonstrate that cell surface expression of both transgenes was at least as high in the clinically normal 21-4L line as in the disease-prone 21-4H line and that endogenous RT1 expression appeared lower in the transgenic rats than in the nontransgenic control. Sources of cell populations were nontransgenic LEW stained with negative control antibody (a), 21-4H (b), 21-4L (c), and nontransgenic LEW stained with anti-RT1 antibody (d). Monoclonal antibodies were anti-HLA-B27 (B.1.23.2) (A), anti-hβ2m (BBM.1) (B), and anti-RT1 class I (OX18) (C).

Table 4. Cell-Mediated Cytotoxicity against HLA-B27

Effector Cells		Effector to Target Ratio	% Cytoxicity of Target Cells	
Donor	Recipient		$B27^+h\beta_2m^+$	$B27^-h\beta_2m^+$
Experiment 1				
21-4L	LEW	100	34	15
		50	38	8
		20	36	3
LEW	LEW	100	19	13
		50	14	7
		20	5	1
Experiment 2				
21-4H	LEW	100	18	7
		50	8	4
		20	3	2
LEW	LEW	100	3	3
		50	1	2
		20	0	1

Spleen cells from LEW rats grafted 7 days earlier with skin from either 21-4 transgenic or normal LEW donors were incubated at the indicated effector target ratios with ^{51}Cr-labeled murine L cell targets expressing either $h\beta_2m$ alone or $h\beta_2m$ and HLA-B27. Incubation times: experiment 1, 6 hr; experiment 2, 4 hr. SD ≤15% in experiment 1 and <10% in experiment 2.

spleen cells from nontransgenic LEW rats receiving grafts from either 21-4H or 21-4L donors showed significantly higher lytic activity against L cell targets transfected with the B27 gene than against otherwise identical targets lacking this gene. Lytic activity was also higher in recipients of transgenic grafts than in recipients of control nontransgenic syngeneic grafts. These results indicate that the B27 transgene product is recognized in a conventional manner by allogeneically primed cytolytic T cells.

Inflammatory Disease in the 21-4H Line: Clinical and Histologic Findings

Gastrointestinal Tract

Overt disease appeared in all of the rats bearing the 21-4H transgene locus that survived past 10 weeks of age. This cohort consisted of 14 males and 9 females. The most common and persistent finding was diarrhea, manifested by frequent, voluminous, often watery stools. Diarrhea was observed in all 23 animals, with equal persistence and severity in the two sexes. Histologically, the gastrointestinal disease was manifested by chronic inflammation involving the stomach and small and large intestine (Figure 3). The distribution and severity of the lesions varied, the colon being the most consistently and prominently affected site. Less frequently, gastric lesions predominated. In all sites, the inflammatory cells consisted primarily of large and small lymphocytes, plasma cells, and smaller numbers of eosinophils. Although the inflammatory response remained primarily in the lamina propria, in the most severely affected regions it extended into the submucosa. Lymphocytes wer commonly aggregated into small hyperplastic lymphoid foci, esp cially in th colon and ileum.

In th intestinal lesions, hyperplasia of crypt epithelial cells replaced mucus-secreting cells and increased the depth of the crypts (Figures 3D and 3F). Hyperplastic crypt cells showed regenerative atypia and a marked increase in mitotic activity. Destruction of crypts and/or the formation of crypt abscesses was uncommon and se n only in the most inflamed areas.

The gastric lesions generally consisted of widely scattered inflammatory foci in the lamina propria and submucosa, but in more severe lesions inflammation was much more extensive, and inflammatory cells accumulated in ectatic glands. The proliferation of mucus-neck cells resulted in marked reduction in the number of parietal cells (Figure 3B).

That the gastrointestinal inflammation did not result from a contagious pathogen was suggested by four pieces of evidence. Stool cultures for aerobic bacteria yielded only normal fecal flora. Furthermore, rats of the 21-4L line and nontransgenic LEW rats were housed for long periods in the same cages with affected 21-4H rats without showing any diarrhea or other signs of illness. In addition, the histology of the gastrointestinal tract of the affected 21-4H rats was not consistent with any known infectious process. Finally, diarrhea has also appeared in six out of seven transgenic rats of the 33-3 line past the age of 2 months, and not in their nontransgenic littermates.

Peripheral and Axial Joints

Peripheral arthritis was observed in 10 of 14 21-4H males and in 1 of 9 21-4H females. This was manifested in most cases by swelling, erythema, and tenderness of the tarsal joints of one or both hindlimbs (Figure 4B). In a few animals the carpal joints or digits were also inflamed (Figure 4D). The arthritis persisted from a few days to several weeks, and in some cases showed an undulating pattern of remission and exacerbation.

Histologically, large accumulations of neutrophils were present in the joint space. The synovium was hyperplastic, edematous and infiltrated with large numbers of lymphocytes, plasma cells, and neutrophils, with neutrophils predominating in the most active lesions (Figure 6B). There was marked pannus formation that eroded the bone at the synovial recess, invading and destroying the articular cartilage. Where the articular cartilage on adjacent joint surfaces was completely replaced by pannus, fibrous ankylosis occurred. Reactive bone formed small osteophytes along the diaphyses, and foci of metaplastic bone were seen within the fibrotic joint capsule. Chronic inflammation extended from the joint capsule to involve adjacent ligaments and tendons. Despite extensive joint destruction evident histologically, resolution generally occurred with preservation of mobility in the large joints.

Vertebral joints from two tails of 21-4H rats were examined histologically, and both revealed inflammatory changes at the outer aspects of the annulus fibrosus and its attachment to the vertebral endplate (Figure 6D). The inflammatory cells consisted of lymphocytes and small numbers of plasma cells mixed with active fibroblasts. There was active bone resorption at the insertion of th annulus and the adjacent periost um was r active.

Skin and Nails

Several animals of both sex s develop d grossly evid nt

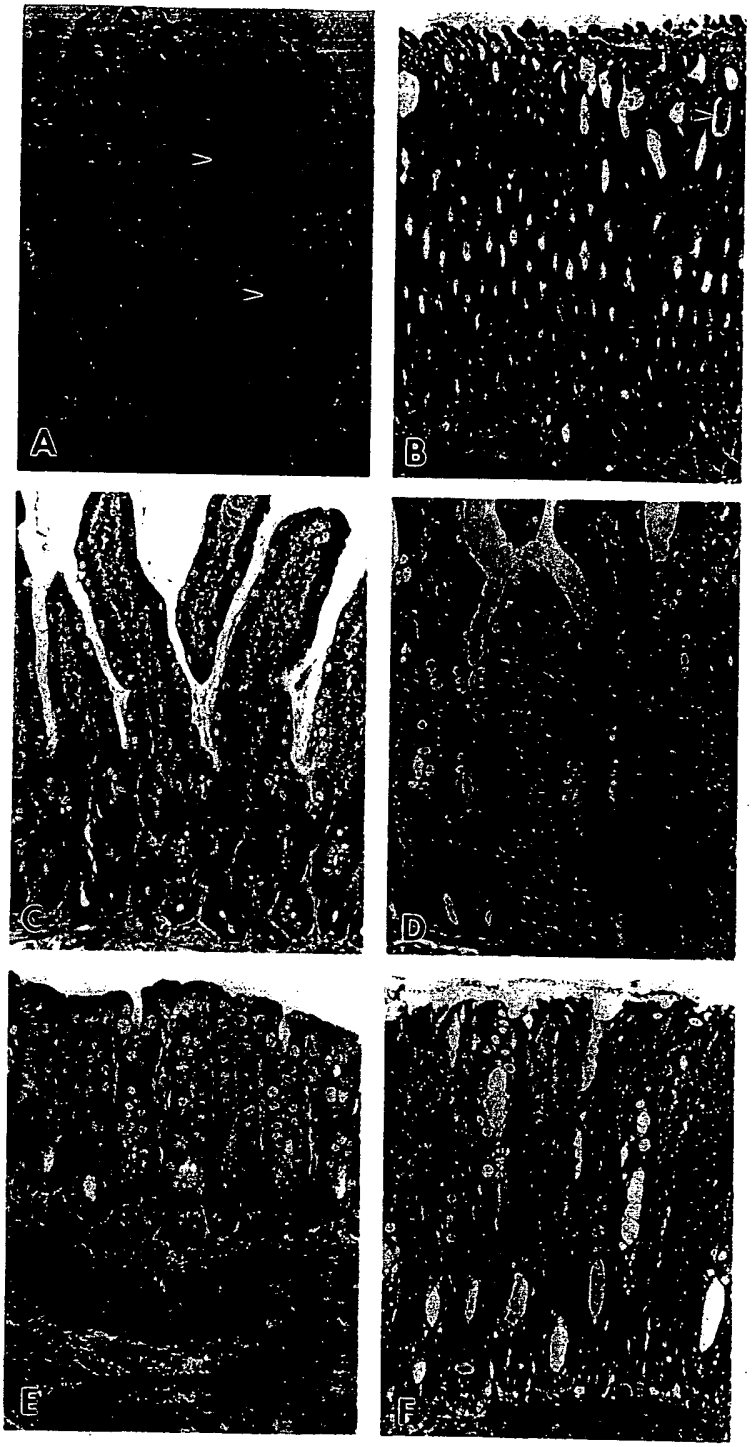

Figure 3. Gastrointestinal Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.
(A) Normal stomach. Arrowheads indicate typical parietal cells (81.25×).
(B) Stomach of a 3-month-old 21-4H male, showing chronic gastritis, with numerous dilated pits and glands (asterisks). A microabscess is present in one dilated gland (arrowhead). Hyperplasia of the mucus-neck cells has largely replaced the parietal cells, and an inflammatory infiltrate is present throughout the lamina propria (65×).
(C) Normal ileum (84.5×).
(D) Ileum of a 3-month-old 21-4H male, showing chronic enteritis. The depth of the crypts is increased due to epithelial cell hyperplasia. There is a loss of mucus-secreting cells, and an inflammatory infiltrate is present throughout the lamina propria (84.5×).
(E) Normal colon (97.5×).
(F) Colon of a 3-month-old 21-4H male, showing chronic colitis. The depth of the crypts is markedly increased due to epithelial cell hyperplasia. There is a loss of mucus-secreting cells, and an inflammatory infiltrate is present throughout the lamina propria (97.5×).

chang s in the tail skin and/or dramatic hyperkeratosis and dystrophy of the nails on all four extremities (Figures 5B and 5D). Histologically, in the tail l sions th epidermis was massiv ly thick ned by psoriasiform hyperplasia (Figur 6F). The rete ridges were r gular and thickened at the base. Exocytosis of lymphocytes and n utrophils was common, with these cells accumulating in spongiotic foci in the epidermis, in the superficial parakeratotic crust, or around d generated, necrotic keratinocytes. Diffuse orthokeratotic hyperkeratosis was prominent. The sup rfi-

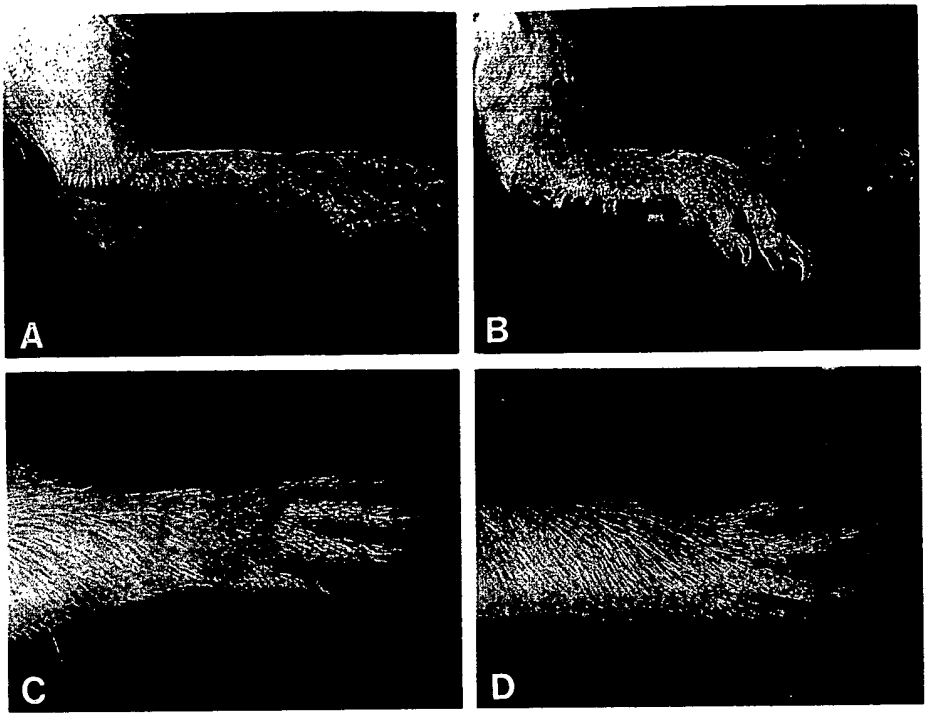

Figure 4. Peripheral Joint Gross Pathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.
(A) Normal distal hindlimb.
(B) Distal hindlimb of a 6-month-old 21-4H male showing swelling and erythema.
(C) Normal distal forelimb.
(D) Distal forelimb of a 4-month-old 21-4H male showing swelling and erythema surrounding the carpal joint.

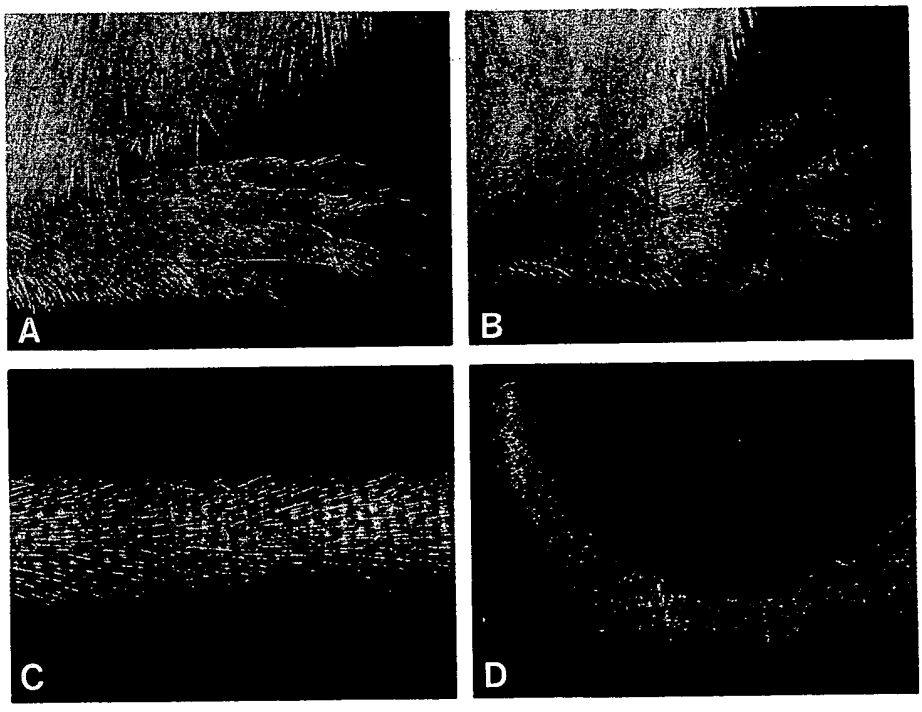

Figure 5. Nail and Skin Gross Pathology of 21-4H Rats

(A) Normal hindlimb digits and nails.
(B) Hindlimb digits and nails of a 3½-month-old 21-4 male, showing hyperkeratosis and dystrophy of the nails and alopecia over the digits.
(C) Normal tail.
(D) Tail of a 3½-month-old 21-4 male (same as in [B]), showing edema, alopecia, flaking, and masking of the normal ridged pattern.

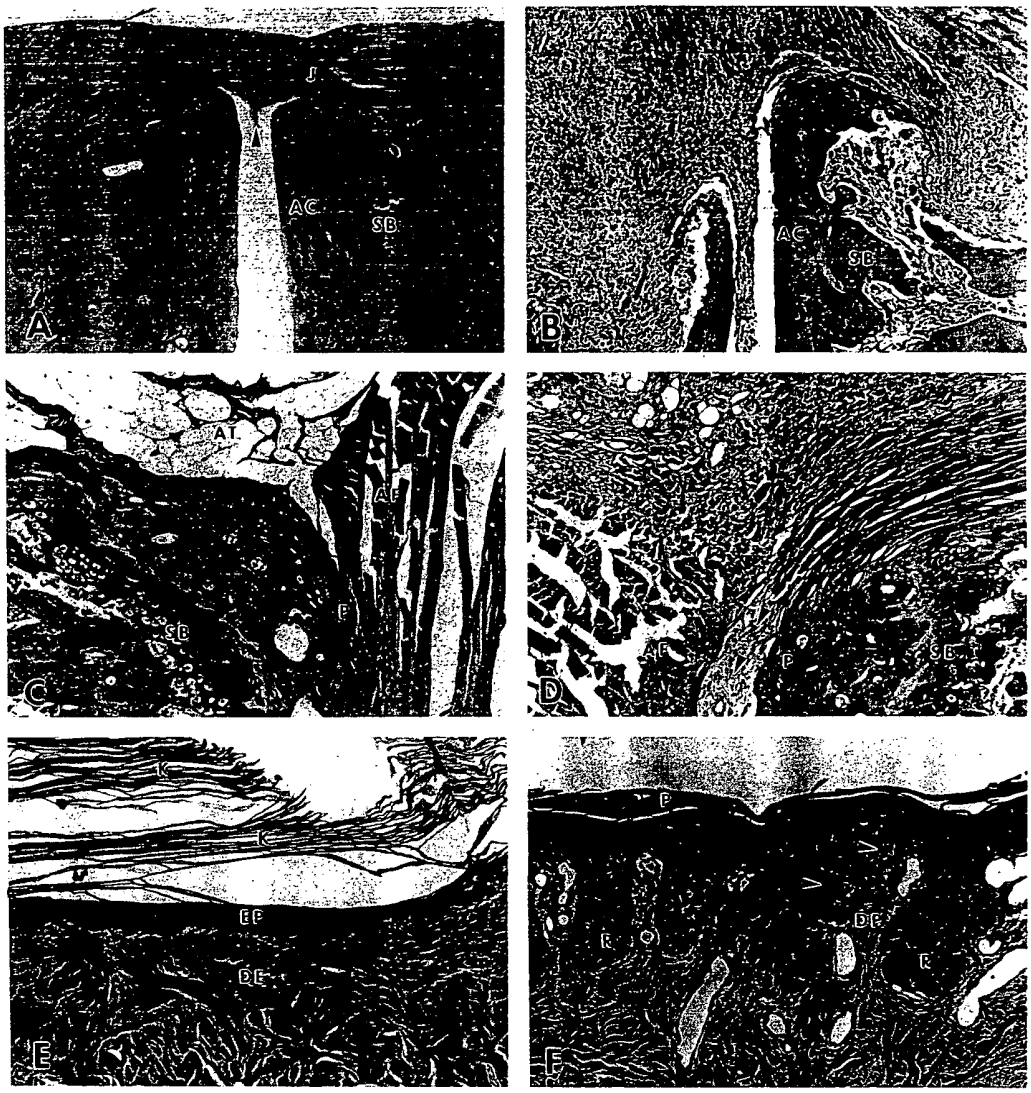

Figure 6. Peripheral and Axial Joint and Skin Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.

(A) Normal tarsal joint. Synovium (arrowhead), articular cartilage (AC), subchondral bone (SB), and joint capsule (J) are labeled (78×).

(B) Tarsal joint of a 4-month-old 21-4H male (same as in Figure 4D), showing chronic arthritis. There is a marked inflammatory infiltrate in the joint capsule and synovium, with pannus (asterisks) eroding articular cartilage (AC) and subchondral bone (SB) on both sides of the joint (58.5×).

(C) Normal tail intervertebral joint. The annulus fibrosus (AF), vertebral end plate (P), ossification center of subchondral bone (SB), and periarticular adipose tissue (AT) are labeled (65×).

(D) Tail intervertebral joint of a 4-month-old 21-4H male (same as in Figure 4D), oriented as a mirror image of (C), showing expansion of the periarticular connective tissue by mononuclear inflammation and fibrosis (asterisks), invading and disrupting the attachment of the outer layers of the annulus to the vertebral end plate (arrowheads). Annulus fibrosus, vertebral endplate, and subchondral bone are labeled as in (C) (58.5×).

(E) Normal tail skin. The keratin layer (K) overlies the epidermis (EP) and dermis (DE) (97.5×).

(F) Tail skin of a 3½-month-old 21-4 male (same as in Figure 5B), showing prominent, elongate, regular rete pegs (R) (psoriasiform epidermal hyperplasia), exocytosis of lymphocytes and neutrophils (arrowheads), parakeratosis (P), and dermal papillae (DP) containing inflammatory infiltrates (78×).

cial papillary dermis contained a diffuse infiltrate of neutrophils, lymphocytes, and plasma cells. Similar changes were seen in skin over the distal aspect of the digits.

Testis and Epididymis

Orchitis and epididymitis were prominent findings in the 21-4H males. The orchitis was manifested clinically by a progressive enlargement of the testes followed by testicular atrophy, with infertility ensuing by 3 months of age in most of the males. In contrast, the females showed little loss of fertility, even in the presence of persistent diarrhea. Histologically, the testicular tunica was thickened by connective tissue, which contained active angioblasts and fi-

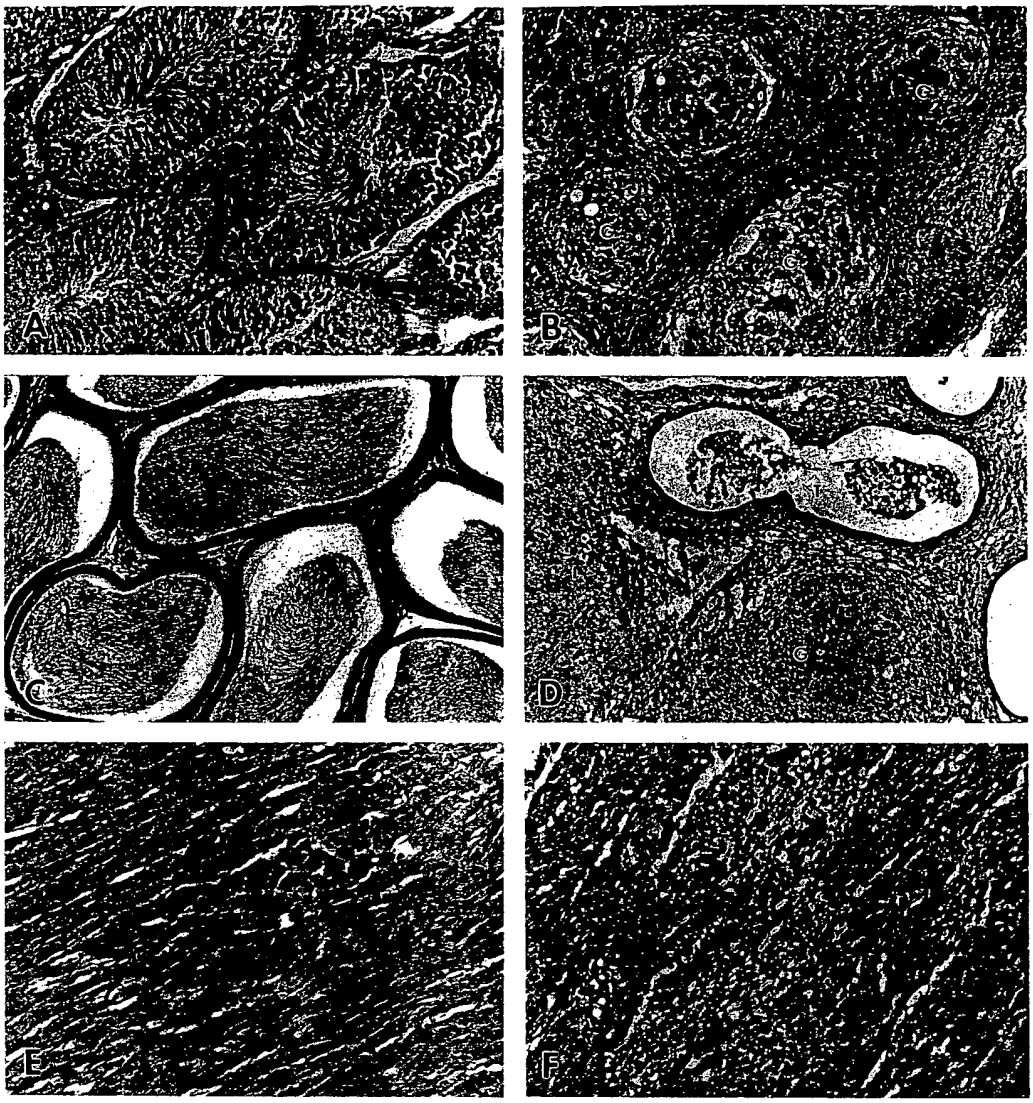

Figure 7. Male Genital Tract and Myocardial Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.

(A) Normal testis (71.5×).

(B) Testis of a 3-month-old 21-4H male, showing chronic orchitis with an intense mononuclear cell interstitial inflammatory infiltrate and sperm granulomas (G) (71.5×).

(C) Normal epididymis (65×).

(D) Epididymis of a 3-month-old 21-4H male, showing chronic epididymitis with a granulomatous interstitial inflammatory cell infiltrate (I), sperm granuloma (G), and dilated tubules (T) containing degenerated inflammatory cells and no sperm (65×).

(E) Normal myocardium (78×).

(F) Myocardium of a 3-month-old 21-4H male (same as in Figure 3F), showing myocarditis with a prominent mononuclear inflammatory cell infiltrate separating the myofibers (78×).

broblasts as well as large numbers of lymphocytes and plasma cells. The testes often contained numerous granulomas with necrotic centers surrounded by epithelioid macrophages and giant cells and peripherally by lymphocytes, plasma cells, and fibrosis (Figure 7B). Central infarction of the testis was a common finding in the most sev rely affected specimens.

The pididymis frequently contained granulomas similar to those found in th testis, along with dilated tubules containing necrotic cellular debris. The interstitium of the epididymis was expanded by lymphocytes, plasma cells, epithelioid macrophages, and moderate fibrosis (Figure 7D).

Heart

Active inflammatory lesions were evident histologically in four of nine 21-4H hearts examined (Figure 7F). In on specimen, extensive multifocal lesions were s n, involving the ventricular walls and septum. Th lesions con-

sisted of large numbers of lymphocytes and small numbers of plasma cells, macrophages, and eosinophils. The myofibers were widely separated by the inflammatory cells, and scattered karyorrhectic nuclei were seen. In the less severely affected specimens, infiltrates of lymphocytes and plasma cells were found at the root of the aortic valve. In more chronic lesions there was moderate fibrosis scattered throughout the myocardium accompanied by mild lymphocytic inflammation. In one animal the adventitia of the great vessels was infiltrated by large numbers of lymphocytes and plasma cells admixed with proliferating angioblasts and fibroblasts.

Eye and Central Nervous System

Mild keratitis and anterior uveitis were observed histologically in one of five eyes from 21-4H rats, one of five eyes from 21-4L rats, and none of four eyes from nontransgenic LEW rats. These findings were judged to be nonspecific, probably secondary to bacterial keratitis.

A peculiar neurologic syndrome was seen in all of the females and most of the males of the 21-4H line. This was manifested by cerebellar ataxia, with intermittent episodes of a stereotypical muscular dystonia, usually in response to handling or some other mild stimulus. Electrophysiologic studies during these episodes demonstrated increased muscular tone without evidence of a cortical seizure focus (data not shown). For several reasons, this abnormality was thought to result from a process distinct from that giving rise to the other lesions. Whereas the other lesions appeared after puberty and then progressed, the neurologic abnormality began within a few weeks after birth and showed no increase in severity thereafter. Unlike the other disease processes, the clinical pattern of the neurologic findings showed little variation from rat to rat. Furthermore, the histologic abnormalities associated with the neurologic disease, which involved primarily the spinal cord and cerebellum, were not inflammatory (data not shown). Finally, there was no evidence of neurologic disturbance in the transgenic F344 line that also showed diarrhea, nor in any of the other transgenic LEW lines.

Other Tissues

The following tissues were examined in at least one of the 21-4H rats showing diarrhea and found not to show histologic abnormalities: esophagus, lung, liver, kidney, adrenal, pancreas, penis, spleen, and thymus. Atrophy of thymus and spleen that was apparent to gross examination was a common finding, however, along with peripheral and mesenteric lymph node enlargement.

Clinical and Histologic Findings in Other Transgenic Lines

No clinical abnormalities were noted in any of the B27 transgenic LEW lines other than 21-4H. Histologic tissue surveys of several 21-4L rats revealed a mild degree of intestinal lymphoid hyperplasia and fibrosis as the only abnormality. Similar intestinal lesions were also found at a low frequency in nontransgenic controls, and hence the significance of these findings in the 21-4L rats is not yet established. As noted above, almost all transgenic rats of the F344 line 33-3 showed diarrhea by 2 months of age.

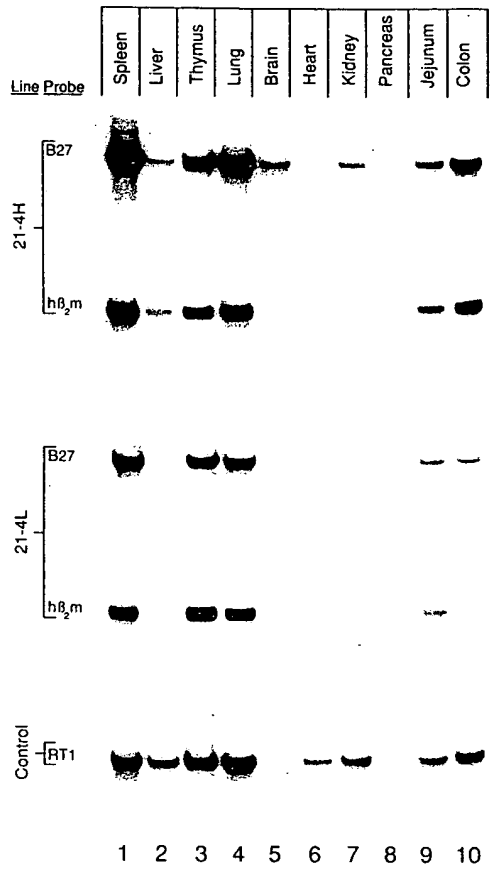

Figure 8. Northern Blot Analysis of HLA-B27, hβ2m, and RT1 mRNA: Tissue Survey

Total cellular RNA from tissues of 12-week-old male 21-4H, 21-4L, and nontransgenic control rats was subjected to denaturing agarose gel electrophoresis (10 μg per lane), transferred to nylon membranes, and hybridized to ^{32}P-labeled probes as described in Experimental Procedures and Figure 1. Membranes were exposed to XAR-5 film at −70°C with intensifying screens for 2–26 hr.

Tissue Distribution of mRNA Expression

Despite the striking differences in disease manifestations, the 21-4H and 21-4L lines showed similar cell surface expression of the transgene products in PBLs (Table 3; Figures 2A and 2B). It was thus of interest to compare the two lines with respect to the level and tissue distribution of mRNA transcripts of both transgenes. Northern blot analysis was carried out on total cellular RNA isolated from tissues of a limited number of rats of the 21-4H and 21-4L lines. HLA-B27 mRNA was detected with a 350 bp probe from the HLA-B 3′ untranslated region (probe C in Figure 1A), and hβ2m mRNA was detected with the same probe used to detect hβ2m genomic DNA (probe D in Figure 1B). RT1 class I mRNA was detected with a 447 bp probe from the 3′ untranslated region of the RT1.A gene. Figures 8 and 9 contain results from age- and sex-matched representatives of the 21-4H and 21-4L lines and a nontransgenic control.

As shown in Figure 8, the distribution and relative abundance of both B27 and hβ2m transgene transcripts among

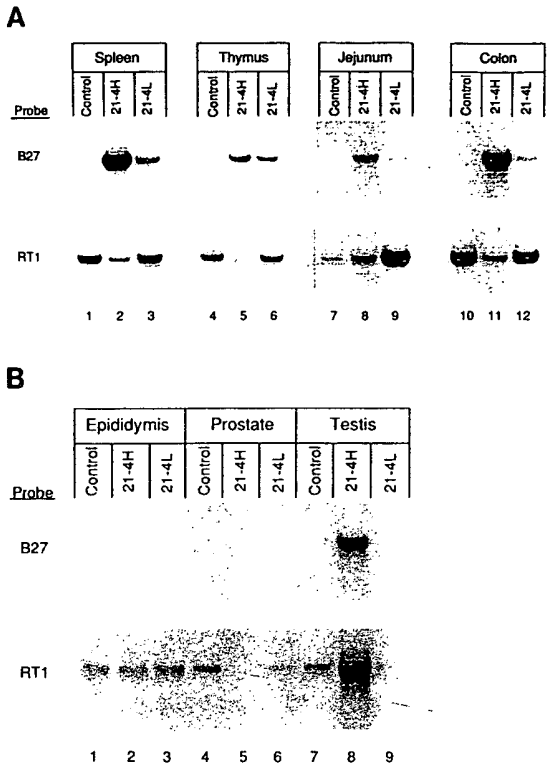

Figure 9. Northern Blot Analysis of HLA-B27 and RT1 mRNA: Comparative Analysis of Seven Tissues

(A) Tissue sources and methods were the same as described in Figure 8. Membranes were exposed 1–8 hr.

(B) Tissue sources were the same as described in Figure 8. Five micrograms of total cellular RNA was added per lane. Prostatic tissue in the 21-4H animal was difficult to identify because of severe atrophy, presumed to be due to loss of androgen stimulation. The membrane probed for B27 was exposed for 1 hr. A 10 hr exposure showed B27 transcripts in 21-4H epididymis (data not shown). The membrane probed for RT1 was exposed for 5 hr.

the various tissues examined were similar to those of the endogenous RT1 class I expression and typical of MHC class I gene expression (Klein, 1986). In addition, both transgenes produced mRNA transcripts of the predicted size.

Figure 9 shows direct comparisons of the 21-4H and 21-4L lines with respect to the relative amounts of B27 and RT1 transcripts in tissues affected by the disease process in the 21-4H line. The abundance of B27 transcripts was dramatically higher in the 21-4H rat than in the 21-4L rat in spleen, colon, and testis, and less markedly increased in jejunum and epididymis. In the thymus, the B27 transcripts were approximately equal in the two lines; however, this may have been a reflection of thymic atrophy in the 21-4H rats.

Although the apparent reduction of RT1 c II surface expression in PBLs was comparable in 21-4H and 21-4L rats (Figure 2C), at the level of mRNA there was no apparent reduction of RT1 transcripts in the 21-4L tissues examined. In contrast, the abundance of RT1 transcripts was markedly reduced in 21-4H spleen, thymus, and colon, compared with tissues from a nontransgenic rat. High expression of RT1 mRNA was found in the 21-4H testis and jejunum. In the case of testis, this probably reflects the intense infiltration of inflammatory cells seen histologically in this organ (Figure 7B), whereas an explanation for the finding in jejunum is less apparent.

Discussion

Integration and Expression of HLA-B27 and $h\beta_2m$ Transgenes in Rats

In an attempt to create an animal model of B27-associated disease, we developed transgenic technology in rats and produced inbred rats expressing both HLA-B27 and $h\beta_2m$. Simultaneously, Mullins et al. (1990), using similar methods, were independently successful in producing transgenic rats expressing a mouse renin gene.

The levels of B27 and $h\beta_2m$ mRNA transcripts in the transgenic tissues paralleled those of the endogenous class I genes in nontransgenic tissues, suggesting that the transgenes were subject to physiologic regulation. It is interesting that the presence of the human transgenes resulted in an apparently reduced expression of the endogenous class I RT1 genes, at the level of cell surface protein expression in PBLs and/or at the level of mRNA, both in lymphoid and nonlymphoid tissue. The possibility was not excluded that the reduced binding of OX18 antibody to the transgenic PBLs was due to an effect of $h\beta_2m$ either on the number of cell surface RT1 class I molecules or on the affinity of the OX18 antibody for these molecules. However, such an effect would not explain the prominent reduction in RT1 mRNA transcripts seen in the 21-4H spleen, thymus, and colon.

Several transcriptional regulatory elements have been identified in the 200 bp 5′ to the transcription initiation site in murine class I MHC genes, including the binding site for the conserved nuclear factor KBF1 (David-Watine et al., 1990; Kieran et al., 1990), and homologous sequences are found in the HLA-B27 promoter region (Weiss et al., 1985). Thus, at least part of the inhibition of RT1 transcription in the 21-4H tissues might be explained by competition by the transgenes for nuclear factor binding.

The Inflammatory Disease of the 21-4H Transgenic Rats: Comparison with B27-Associated Disease in Humans

B27-associated disorders in humans encompass a spectrum of inflammatory diseases affecting predominantly the peripheral and axial musculoskeletal system, gastrointestinal tract, genital tract, integument, and eye (Table 1). Less common involvement of heart and nervous system and rare involvement of lung are also observed in these disorders (Bulkley and Roberts, 1973; Good, 1974; Taurog and Lipsky, 1990). The spontaneously arising disease in B27/$h\beta_2m$ transgenic rats showed a striking clinical and histologic similarity to B27-associated disease in humans, with inflammatory lesions of peripheral and axial joints, gut, male genital tract, nails, skin, and heart. The close resemblance of the findings in the transgenic rats

to B27-associated disease in humans strongly supports the conclusion that the B27 molecule itself participates in the pathogenesis of the various lesions found in different organ systems in the spondyloarthropathies.

The most prevalent site of inflammation in the transgenic rats appears to be the gastrointestinal tract. All of the 21-4H rats under observation for at least 6 months developed overt diarrhea, and a similar picture is emerging in the 33-3 line. These findings suggest that the events initiating the disease process occur in the gastrointestinal tract and that further investigation of the intestinal immunophysiology and immunopathology of the transgenic animals may provide some insight into the role of the B27 molecule in these events.

Numerous observations in humans support a causal link between factors in the gut and inflammatory joint disease. Peripheral and axial arthritis are common accompaniments of chronic inflammatory bowel disease even in the absence of B27 (Table 1), and recent evidence suggests that milder degrees of gastrointestinal inflammation are closely correlated with the occurrence of B27-associated joint disease in individuals without bowel symptoms. Histologic examination of endoscopically obtained biopsies in a large series of patients with reactive arthritis or ankylosing spondylitis indicated that over 60% had asymptomatic inflammatory lesions of the terminal ileum or colon (Cuvelier et al., 1987). Whether patients with B27-associated disease develop inflammatory lesions in the more proximal small intestine or stomach that might resemble those seen in the 21-4H rats is not known.

Although gastrointestinal inflammation in the transgenic rats was present equally in both sexes, arthritis occurred predominantly in males. This closely followed the pattern in humans, in whom males with ankylosing spondylitis, juvenile onset spondyloarthropathy, or reactive arthritis following genital infection outnumber females 3- to 10-fold. The prevalence of subclinical gastrointestinal inflammation in B27 individuals without rheumatic disease, either male or female, is not known. Both peripheral and axial arthritis occurred in the 21-4H rats. Clinically, the peripheral arthritis resembled that seen in other experimental models of arthritis in rats, such as those induced by complete Freund's adjuvant or streptococcal cell walls, with swelling and erythema of the proximal hind paw being the predominant lesion. Histologically, the involved joints showed lesions typical of experimental arthritis in rats, as well as B27-associated peripheral arthritis in humans, with synovial hyperplasia, inflammatory cell infiltration, pannus formation, and destruction of articular cartilage and bone (Greenwald and Diamond, 1988; Taurog et al., 1988b).

Axial arthritis, with inflammatory cell infiltration and periosteal reaction at the margins of the intervertebral discs, was seen histologically in the tails of 21-4H rats. This appears to be the same pathologic process that leads to the vertebral changes in ankylosing spondylitis, although histologic comparison of this lesion with human spondylitis is made difficult by the paucity of descriptions of arly lesions in humans (Ball, 1971; Eulderink, 1990). More generally, the vertebral lesion in the 21-4H rats also closely resembles the enthesitis, inflammation at ligamentous attachments to bone, that is a pathologic hallmark of the B27-associated diseases in humans (Ball, 1971).

Dramatic psoriasiform skin and nail lesions developed in the 21-4H rats. These lesions show an extraordinary histologic resemblance to psoriatic lesions in humans. Although in most patients with psoriasis vulgaris there is no association with HLA-B27, lesions termed keratoderma blenorrhagica that are histologically indistinguishable from the psoriatic variant pustular psoriasis are commonly found in B27-associated reactive arthritis (Good, 1974; Keat, 1983). Furthermore, typical psoriasis vulgaris occasionally supervenes in patients initially presenting with reactive arthritis. Finally, a common pathogenetic mechanism between psoriasis vulgaris and B27-associated disease is suggested by the recent observation that both psoriasis vulgaris and the skin lesions of Reiter's syndrome appear to be significantly exacerbated in patients with coexistent infection with the human immunodeficiency virus HIV-1 (Duvic et al., 1987).

Another striking lesion in the 21-4H rats was orchitis, which was found in virtually all of the males, invariably in association with epididymitis. In humans, urogenital inflammation is prevalent in B27-associated diseases. Although urethritis in males with reactive arthritis is a common finding even in the absence of known urethral infection, prostatitis and epididymitis in males, cervicitis in females, and cystitis in both sexes have been described (Yli-Kerttula, 1984). Although there have been no reports of histologically confirmed orchitis associated with HLA-B27 or with B27-associated syndromes, clinical descriptions suggestive of orchitis have been published (Montanaro and Bennett, 1984). It is thus not altogether unlikely that the inflammatory process induced by B27 in the 21-4H rat testis has a milder human counterpart.

Inflammatory disease involving the root of the aortic valve and myocardium was found in the 21-4H rats. Both aortic insufficiency and cardiac conduction disturbances are well-documented complications of ankylosing spondylitis and reactive arthritis (Bergfeldt et al., 1988; Bulkley and Roberts, 1973; Good, 1974). Moreover, primary myocardial disease may also be relatively prevalent in ankylosing spondylitis (Brewerton et al., 1987). The cardiac pathology of the 21-4H rats, like the lesions in the peripheral and axial joints, gastrointestinal tract, skin, and male genital tract, thus appears to be a direct counterpart of a pathologic process in B27-associated human disease.

In comparing the pathologic lesions identified in the B27 transgenic rats with B27-associated disease in humans, only the neurologic disease in the 21-4H LEW line seemed to represent a significant anomaly. Occasional cases of either central or peripheral neurologic disease have been reported in association with B27-associated reactive arthritis (Good, 1974; Montanaro and Bennett, 1984; Taurog and Moore, 1986), but none of these has been characterized histologically, nor do their clinical descriptions resemble the findings in the 21-4H rats. As mentioned under Results, the neurologic lesions in the 21-4H rats appear to be temporally and histologically unrelated to the inflammatory disease seen in other organs.

Although the possibility cannot be excluded that the neurologic disturbance contributed indirectly to the inflammatory lesions, for example by disruption of the normal innervation of lymphoid tissue or gut (Anderson, 1990), the absence of neurologic disease in the 33-3 line, a second transgenic line exhibiting spontaneously occurring B27-associated disease, suggests that the neurologic disease in the 21-4H line is not a necessary part of the inflammatory process in other organ systems, but likely a result of a dominant insertional mutation. A complete description of the neurologic findings in the 21-4H line is in progress.

The Inflammatory Disease of B27/hβ_2m Transgenic Rats: Possible Mechanisms

It is unclear why overt inflammatory disease developed in only two of the seven transgenic rat lines, 21-4H and 33-3. It is unlikely that differences in postconceptional environment play a significant role in determining the phenotypes of the different transgenic lines, since segregation of the diseased phenotype with the 21-4H locus was uniformly observed in litters containing both 21-4H and 21-4L offspring. Insertional mutation appears unlikely as an explanation, since two independent transgenic lines developed aspects of a similar disease. Nor was evidence obtained for differences in B27 function, since the 21-4H and 21-4L lines comparably stimulated immune recognition of B27 by cytolytic T cells. The variation among transgenic rat lines most likely can be ascribed to either quantitative or qualitative differences in the expression of the transgenes or to differing effects of the transgene on the host genome.

The results presented in this study do not exclude the possibility that a human class I MHC gene other than HLA-B27 might also be capable of producing a disease process similar to that described here, nor do they exclude the possibility that the hβ_2m gene alone might be sufficient to produce disease. Studies are in progress to address these possibilities.

Several lines of evidence have suggested that interactions between B27 and bacterial products are involved in the pathogenesis of the spondyloarthropathies (Yu et al., 1989). Although the disease in the transgenic rats arose spontaneously in the apparent absence of infection by pathogens, the possibility must be considered that the pathogenesis involves interactions between B27 and commensal organisms such as the intestinal flora or pathogens not detected by routine serologic screening. Studies in which the transgenic rats are maintained germ free will be important in exploring this issue.

Despite extensive investigation of the structure and function of class I MHC genes in general and HLA-B27 in particular, it has so far not been possible to identify the molecular mechanism of the association of B27 with human disease. However, given the close resemblance of the spontaneous disease of the 21-4H line to B27-associated human disorders, a detailed cellular and molecular analysis of th B27/hβ_2m transgenic rats should enhance our understanding of the role of HLA-B27 in causing dis- as . It may also contribute to a broad r understanding of th function of class I MHC molecul s.

Experimental Procedures

Animals

Specific pathogen-free inbred Lewis/CrlBR (LEW) and Fischer F-344/CrlBR (F344) rats, and outbred Sprague-Dawley rats, were purchased from Charles River Laboratories, Boston, MA. Hybrid mice of the transgenic line 56-3 (Taurog et al., 1990), which express high levels of both B27 and hβ_2m on lymphoid cell surfaces, were bred in our animal colony. Animals were maintained in accordance with institutional guidelines.

Generation and Identification of Transgenic Rats

Immature LEW or F344 female rats were superovulated according to the method of Armstrong and Opavsky (1988) and bred with fertile males. The day following breeding, fertilized one-cell eggs were flushed from the oviduct of females exhibiting either vaginal plugs or sperm in vaginal lavage fluid. Eggs were held in Brinster's medium for 2 hr or less before microinjection. Microinjection of eggs and transfer to day 1 pseudopregnant Sprague-Dawley females were carried out essentially as described for mice (Brinster et al., 1985).

Two genomic clones were used for microinjection of fertilized rat eggs (Figure 1). The HLA-B27 gene encoding the HLA-B* 2705 subtype (Bodmer et al., 1990) was contained on a 6.5 kb EcoRI fragment (clone pE.1-B27; Taurog et al., 1988a; Taurog and El-Zaatari, 1988) and the hβ_2m gene was contained on a 15 kb SalI–PvuI fragment (clone pβ2m-13, the gift of Dr. H. L. Ploegh, Amsterdam, The Netherlands; Güssow et al., 1987). Each insert was separated from plasmid DNA by agarose gel electrophoresis and isolated by perchlorate elution (Chen and Thomas, 1980). The solution used for microinjection contained both fragments, each at 1.5 ng/μl.

Identification and quantitation of transgenes were determined in the founder animals and their progeny by dot-blot hybridization of genomic DNA isolated from tail biopsies, as previously described (Brinster et al., 1985). Genomic DNA was analyzed by hybridization with 5′ and 3′ flanking probes for the *HLA-B* locus, as previously described (probes A and B in Figure 1A; Taurog et al., 1988a), and with a 3.7 kb BglII fragment containing exons 2 and 3 of the hβ_2m gene (probe D in Figure 1B).

RNA Analysis by Northern Blot Hybridization

Northern blot hybridization was carried out as described elsewhere (S. D. Maika, L. Laimonis, A. Messing, and R. E. Hammer, submitted). Briefly, total cellular RNA was extracted from tissues by the guanadinium isothiocyanate–CsCl procedure, separated on glyoxal agarose gels, and blotted onto nylon membranes. HLA-B27 mRNA was detected with the 350 bp HLA-B 3′ untranslated region probe pHLA-1.1 (probe C in Figure 1A; Koller et al., 1984), and hβ_2m mRNA was detected with the same 3.7 kb BglII fragment used to detect hβ_2m genomic DNA (probe D in Figure 1B). RT1 class I mRNA was detected with a 447 bp PvuII–HindIII fragment containing the 3′ untranslated region of the RT1.A^a gene pBS3.3/1 (the gift of Dr. J. C. Howard, Cambridge, England; Rada et al., 1990). All stringency washes were carried out in 0.1× SSC, 0.5% SDS at 65°C.

Monoclonal Antibodies, Indirect Immunofluorescence, and Flow Cytometry

The following murine monoclonal antibodies were used: B.1.23.2, IgG_{2b}, binding a monomorphic determinant shared by HLA-B and -C molecules (Rebai and Malissen, 1983); BBM.1, IgG_{2b}, binding hβ_2m (Brodsky et al., 1979); and OX18, IgG_1, binding a monomorphic rat RT1 class I antigen (Fukumoto et al., 1982). P1.17, an IgG_{2a} myeloma, served as a negative control.

Indirect immunofluorescence was carried out as previously described (Taurog and El-Zaatari, 1988; Taurog et al., 1988a). Briefly, Ficoll–Hypaque-purified peripheral blood mononuclear cells were incubated with saturating concentrations of each monoclonal antibody, washed, then incubated with fluorescein-conjugated F(ab)′$_2$ fragments of goat anti-mouse Fcγ antibodies (Cappel Inc., Malvern, PA). After washing, the cells were fixed in 1% paraformaldehyde before analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Viable lymphocytes were analyzed by gating of forward and 90° light scatter.

Generation and Analysis of Cytolytic T Cells

Primary alloimmunization by skin grafting was carried out by the method of Peter and Feldman (1972). Seven days after graft placement, recipient spleen cells were used as effector cells in a 4–6 hr ^{51}Cr release assay, as previously described (Taurog et al., 1988a). Two mouse L cell lines were used as target cells, one transfected with and expressing the hβ2m gene, the other transfected with and expressing both the HLA-B*2705 and hβ2m genes, as previously described (El-Zaatari et al., 1990).

Histology

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Joints were embedded and sectioned following fixation and decalcification for 4–6 weeks in 10% disodium EDTA, as previously described (Taurog et al., 1988b), or following decalcification in 10% formic acid. Eyes were embedded in methacrylate before sectioning and staining.

Acknowledgments

The technical contributions of Jana L. Hlavaty and Beverly Y. Hastings are gratefully acknowledged. The authors thank Dr. David T. Armstrong for providing data prior to publication; Dr. Jonathan C. Howard for providing the pBS3.3/1 gene and data prior to publication; Dr. Jerry Y. Niederkorn and Marsha S. Pidherney for assistance with histologic sections of eyes; Dr. Albee Messing and Dr. Gerald A. Marks for assistance with preliminary characterization of the neurologic disturbance in the 21-4H line; and Dr. Joseph L. Goldstein, Dr. Michael S. Brown, and Dr. Peter E. Lipsky for helpful discussions and critical review of the manuscript. R. E. H. and J. D. T. also wish to thank Dr. Lipsky for encouraging us to undertake this project. This work was supported by NIH grants AR09989 (J. D. T. and R. E. H.) and RR00890 (J. A. R.) and a grant from the North Texas Chapter of the Arthritis Foundation (J. D. T. and R. E. H.). J. D. T. was supported by NIH Research Career Development Award AR01756 during part of this work.

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Received October 8, 1990.

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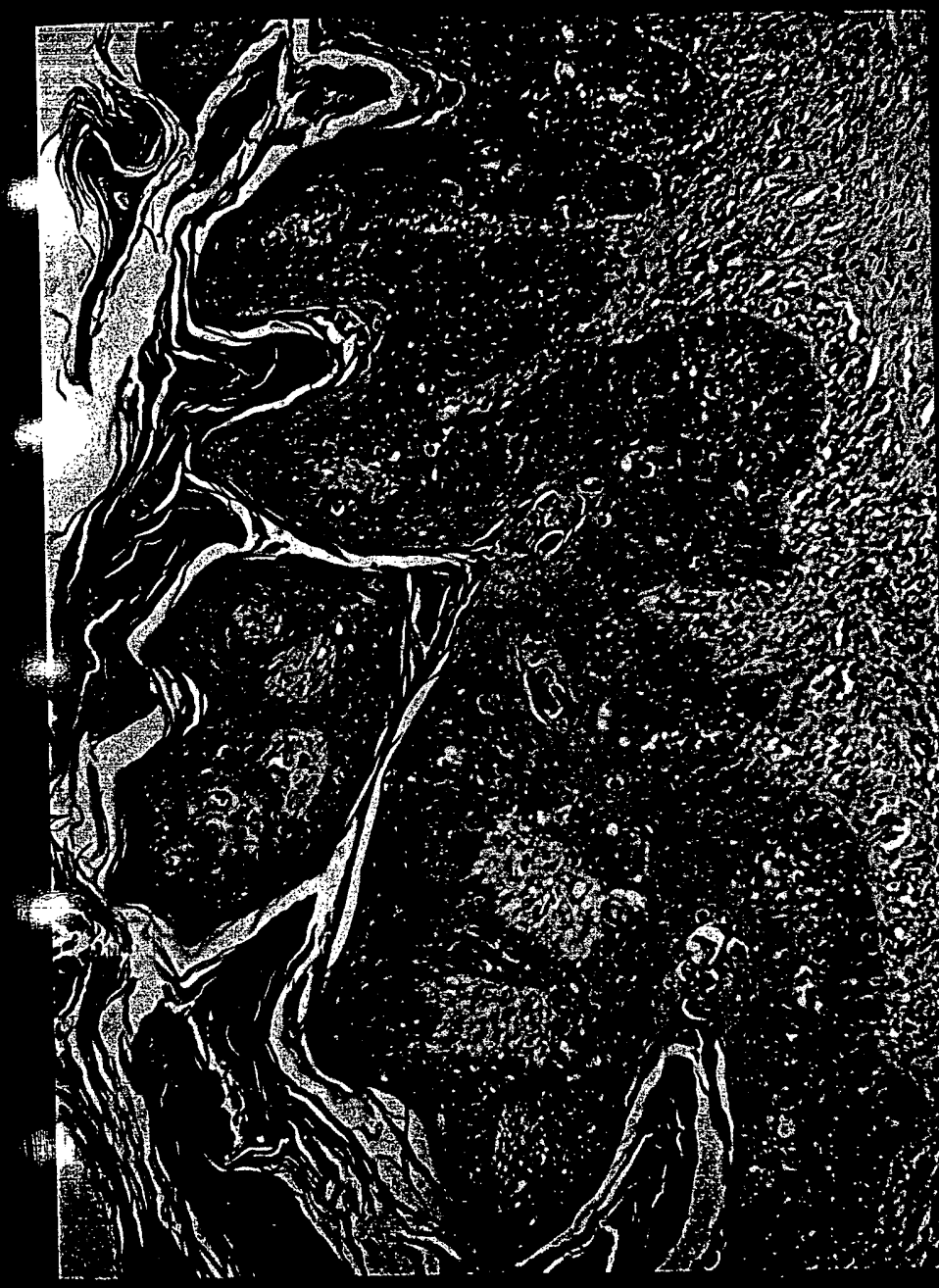
Cell

Volume 63 Number 5 November 30, 1990



Human HLA-B27 Causes Spondyloarthropathies in Transgenic Rats

Physiological characterization of the hypertensive transgenic rat TGR(mREN2)27

MIN AE LEE, MANFRED BÖHM, MARTIN PAUL,
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Lee, Min Ae, Manfred Böhm, Martin Paul, Michael Bader, Ursula Ganten, and Detlev Ganten. Physiological characterization of the hypertensive transgenic rat TGR(mREN2)27. *Am. J. Physiol.* 270 (*Endocrinol. Metab.* 33): E919–E929, 1996.—Transgenic techniques represent powerful tools for the study of gene-related mechanisms of diseases such as hypertension, which results from a complex interaction between genetic and environmental factors. The renin-angiotensin system, a biochemical cascade in which renin functions as the key enzyme in the formation of the effector peptide angiotensin II, plays a major role in the regulation of blood pressure. The renin gene, therefore, represents an important candidate gene for hypertension. Because rats are more suited than mice for a number of experimental settings often employed in cardiovascular research, we modified the transgenic technique to generate the transgenic rat strain TGR(mREN2)27 harboring the murine *Ren*-2 gene. These transgenic rats develop fulminant hypertension at an early age despite low levels of renin in plasma and kidney. In addition, high expression of the transgene in a number of extrarenal tissues is associated with increased local formation of angiotensin II. Thus the TGR(mREN2)27 rat represents a model of hypertension with a defined genetic background. Studies on the transgenic rat may not only provide new insights into pathophysiological mechanisms of hypertension in this animal model but also offer the unique possibility to investigate the function and regulation of renin-angiotensin systems in extrarenal tissues. The aim of this review is to compile the knowledge that has been accumulated to date on this transgenic rat and to discuss possible mechanisms responsible for its hypertensive phenotype.

renin-angiotensin system; hypertension

MOST KNOWLEDGE concerning the pathophysiology of primary hypertension has largely been obtained from studies performed in animal models such as the spontaneously hypertensive rat (SHR) and its stroke-prone substrain (SHRSP), which were established by selective breeding of the hypertensive phenotype. As in most cases of primary hypertension in humans, high blood pressure in these animal models is a quantitative trait under polygenic control (61). Apart from rare monogenetic forms of hypertension (43, 70), the genes involved in the hypertensive process remain largely unknown. One approach to identify chromosomal regions containing such genes is to perform linkage studies in crosses between hypertensive rat strains and their normotensive control strains (44, 61). Another approach is to analyze mechanisms by which selected genes influence the development of pathophysiological phenotypes with the use of transgenic techniques (20). The major advantage of transgenic animal models over disease models of unknown etiology is that ensuing pleiotropic phenotypic effects can be attributed to a single genetic change. This is especially important in the study of complex diseases such as hypertension, in which causal effects are difficult to differentiate from secondary effects.

The renin-angiotensin system (RAS) is pivotally involved in the regulation of blood pressure and fluid homeostasis. Therefore, the genes encoding for its components represent important candidate genes for hypertension. In addition to its well-defined role as an endocrine system, there is increasing evidence for locally acting RAS in several tissues, including brain (60), adrenal glands (90), kidney (42), heart (45), and reproductive organs (15). Because renin represents the key enzyme in the cascade leading to the generation of angiotensin II

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(ANG II), the murine *Ren*-2 gene was chosen as a candidate gene to generate transgenic rats (51).

TRANSGENIC METHODOLOGY

Significance of Transgenic Techniques

Since its first application in mammals more than a decade ago, the generation of transgenic animals by introducing foreign genes into a host genome has become a very powerful method in experimental biology and medicine. The transgenic approach, based on gain-of-function alterations, has yielded numerous insights into the physiological role of genes under in vivo conditions. In particular, it has provided unique tools to study the tissue-specific or developmental regulation of genes and to analyze gene-related mechanisms of disease (19, 24). The most commonly used species for the generation of transgenic animals are mice because of the availability of several inbred strains with a well-defined genetic background. Although studies on transgenic mice have provided further insight into the role of certain genes involved in cardiovascular homeostasis, such as vasopressin, atrial natriuretic peptide, and the low-density lipoprotein receptor (19), their small size limits their usefulness for a number of experimental settings often employed in hypertension research. Therefore, we decided to apply the transgenic methodology to the rat. The murine *Ren*-2 gene was chosen for the following reasons. *1*) The renin gene represents a major candidate gene for hypertension on the basis of its physiological and pathophysiological role in the regulation of blood pressure. In addition, allelic variants of the renin gene have been shown to cosegregate with the hypertensive phenotype in certain rat strains (37, 62). *2*) Mouse renin is capable of cleaving rat angiotensinogen to produce angiotensin I (ANG I) and increases blood pressure if injected into rats (55, 56). *3*) The murine *Ren*-2 gene differs from other mammalian renin genes in that its protein is unglycosylated and shows a distinct pattern of extrarenal expression (71). Moreover, introduction of the *Ren*-2 gene into the genome of mice has already been demonstrated to result in correct tissue-specific expression (52). Thus, it was hypothesized that overexpression of murine renin in rats would affect blood pressure and allow investigation of those tissues in which local RAS may play an important functional role.

Generation of the Transgenic Rat Line TGR(mREN2)27

A modified version of the protocol etablished for the generation of transgenic mice by pronucleic injection was applied to the rat (28). Female rats derived from a cross between female outbred Sprague-Dawley (SD) rats (Zentralinstitut für Versuchstierkunde, Hannover) and male Wistar-Kyoto (WKY) rats were subjected to hormonal treatment to induce superovulation. At 9–10 wk of age, rats had osmotic minipumps, loaded with 200 μg of a pregnant mare's serum gonadotropin, implanted subcutaneously. Two days later, the rats were injected intraperitoneally with 30–40 units of human chorionic gonadotropin and mated with male SD rats. The following day, the animals were killed, and fertilized oocytes were harvested for microinjection. After the development of pronuclei, ~1 pl of injection buffer containing foreign DNA at a concentration of 1 ng/μl was injected into the larger male pronucleus by use of microinjection apparatus. The injected DNA contained the entire *Ren*-2 gene of DBA/2 mice, including 5.3 kb of the 5′-flanking and 9.5 kb of the 3′-flanking region (51, 52). Manipulated oocytes were transferred to the oviducts of pseudopregnant SD females by injecting medium containing up to 15 oocytes into the infundibulum via retroperitoneal access. Pseudopregnancy was achieved by mating with vasectomized males. Screening of offspring for successful integration of the transgene was performed by Southern blot analysis of *Pvu* II digested genomic DNA obtained from tail biopsies with use of a radioactively labeled *Bam*H I fragment of the *Ren*-2 cDNA. A transgene-positive offspring was identified by the presence of 8.5-kb and 0.8-kb *Ren*-2-specific restriction fragments (51).

Implantation of 37 eggs resulted in eight progeny, of which five carried the transgene (51). Four of the founder animals were successfully bred, and three of them, TGR (mREN2)25, -26, and -27, transmitted the transgene to their progeny. All transgenic founder animals exhibited blood pressure values in the range of 230–265 mmHg compared with 120 mmHg in transgene-negative littermates. The female founder animal 26 was not hypertensive at onset, and further breeding revealed this animal to be mosaic for the transgene. It was not possible to breed these animals to homozygosity because all male offspring were infertile. In contrast to homologous recombination techniques, insertion of foreign DNA by pronucleic injection does not target a specific chromosomal site and occurs at random. The fact that all transgene-positive founder animals were hypertensive and that the hypertensive phenotype cosegregated with the transgene indicates that hypertension development is independent of the transgene insertion site and not caused by a mutation associated with the integration event. The infertility of male TGR(mREN2)26 may be due either to an insertional mutation on the X chromosome or to an autosomal dominant mutation, because the phenotype is already present in the heterozygous state. The transgenic rat strain TGR(mREN2)27 was established by breeding transgene-positive progeny with SD rats. The founder animals were derived from a cross between a female SD × WKY hybrid and a male SD rat and thus derived one-fourth of their genetic material from the WKY genome. Because further breeding was performed by using SD rats and selecting for the transgene, it can be calculated that the transgene was completely bred into the genetic background of SD rats after 8–10 generations (61). Therefore the SD rat derived from the Hannover colony repesents the appropriate control strain for TGR(mREN2)27 rats.

CHARACTERIZATION OF PHENOTYPE

Development of Blood Pressure, Body Weight, and Survival Rates

The presence of an additional renin gene in TGR(mREN2)27 rats is associated with the development of severe hypertension (51). In heterozygous animals, hypertension was evident at 4–5 wk of age, reaching maximum values of up to 240 mmHg in males and up to 200 mmHg in females at 8–9 wk. Blood pressure values in SD rats ranged between 115 and 140 mmHg (Fig. 1). The phase of established hypertension was followed by a decrease in blood pressure by 20–30 mmHg in male, and by 40–60 mmHg in female TGR(mREN2)27 rats between 20 and 24 wk of age. Doubling of gene dose in homozygous rats led to even higher blood pressure values, reaching 290 mmHg in males and 250 mmHg in females (Fig. 1). In heterozygous transgenic animals, increase in body weight, an indicator of thriving, was comparable to that of SD rats. Homozygous animals, however, displayed a lack of weight gain, which was paralleled by an increased mortality rate (Fig. 2). Morphological changes consistent with hemorrhagic stroke were occasionally found on autopsy predominantly in homozygous TGR(mREN2)27 rats.

Plasma RAS

On the basis of most studies conducted so far on the plasma RAS in the transgenic rat, one of the most interesting observations after the introduction of an additional renin gene in the rat is that renin in plasma and kidney is reduced, whereas prorenin is elevated (51). The suppression of renin and the prorenin elevation were both present at 5 wk of age before hypertension was fully established (Fig. 3, *A-D*). Converting-enzyme activity in plasma was similar between heterozygous TGR(mREN2)27 and SD rats (Fig. 3, *E* and *F*), although homozygosity for the transgene has been shown to be associated with a reduction in plasma-converting enzyme (10, 39). Plasma levels of ANG I were decreased to 20% of control values in transgenic females but were only slightly lower in transgenic than in control males (Fig. 4, *A* and *B*). Similarly, ANG II was reduced in transgenic females compared with female controls but was not different between transgenic and control males (Fig. 4, *C* and *D*). Altogether, ANG I and ANG II were significantly higher in male than in female transgenic rats. This sexual dimorphism was not present in control animals. Plasma angiotensinogen was slightly decreased in both female and male transgenic rats compared with controls (Fig. 4, *E* and *F*).

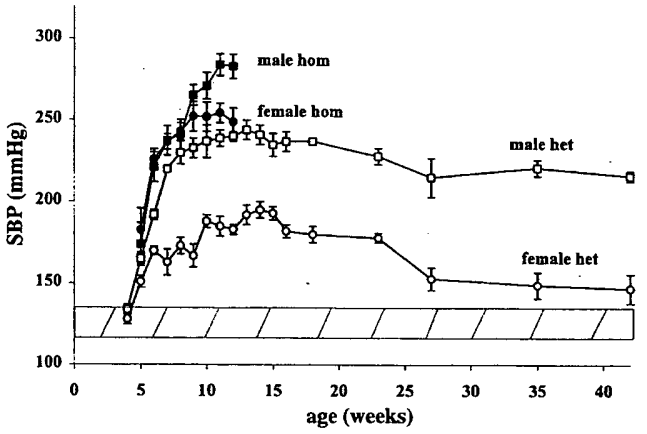

Fig. 1. Development of systolic blood pressure (SBP) measured by tail cuff plethysmography in heterozygous (het) and homozygous (hom) transgenic rat (TGR) (mREN2)27 rats of both sexes. Values are means ± SD; n = 10/group. $P < 0.005$ at 5 wk and $P < 0.0005$ beyond 5 wk of age for all groups vs. normotensive Sprague-Dawley (SD) rats matched for age and sex, respectively, except for female heterozygous rats, $P < 0.0005$ only until 21 wk. Hatched bar, range of blood pressure values in control animals.

Although most investigations on TGR(mREN2)27 rats have confirmed the presence of reduced circulating renin (3, 26, 39, 40, 47, 50, 59, 64, 66), some studies have reported increased plasma renin levels in TGR(mREN2)27 rats (10, 80, 81). This discrepancy clearly requires further exploration, as it concerns the role of circulating renin for the hypertensive phenotype. Several factors must be considered when the levels of circulating renin reported in these studies are interpreted. First, phenotypic effects of the transgene may vary because of differences in the genetic background of the host genome. Whitworth et al. (88) recently demonstrated that crossbreeding of TGR(mREN2)27 rats with Edinburgh SD rats resulted in a change of the phenotype resembling malignant hypertension. Because this phenotype was not observed during crossbreeding of TGR(mREN2)27 rats with Hannover SD rats under identical environmental conditions, the phenotypic shift was attributed to the genetic diversity within outbred SD rats (88). Thus differences in the genetic makeup between different colonies of SD rats may also affect plasma renin levels, emphasizing the need for appropriate controls. Second, plasma renin levels vary depending on gender, age, and zygosity of the transgenic rats. Moreover, high blood pressure in homozygous transgenic rats necessitates antihypertensive treatment during pregnancy. Prenatal treatment with a converting-enzyme inhibitor in SHR has been demonstrated to attenuate development of hypertension in adult rats even after withdrawal of the drug (89). It is possible that similar epigenetic influences in homozygous TGR(mREN2)27 rats may affect the phenotype. Third, prorenin can be activated in vitro after exposure to cold or low pH (54), and artifactual activation during sample processing may account for increased renin, especially in the transgenic rat, where circulating prorenin is high. In addition, species-specific differences in plasma renin levels measured may occur depending on the pH, as well as the source of renin substrate used (23, 81).

Sexual Dimorphism

TGR(mREN2)27 rats exhibit a marked sexual dimorphism with respect to the hemodynamic phenotype because males have higher blood pressure than females. Therefore, the transgenic rat offers the possibil-

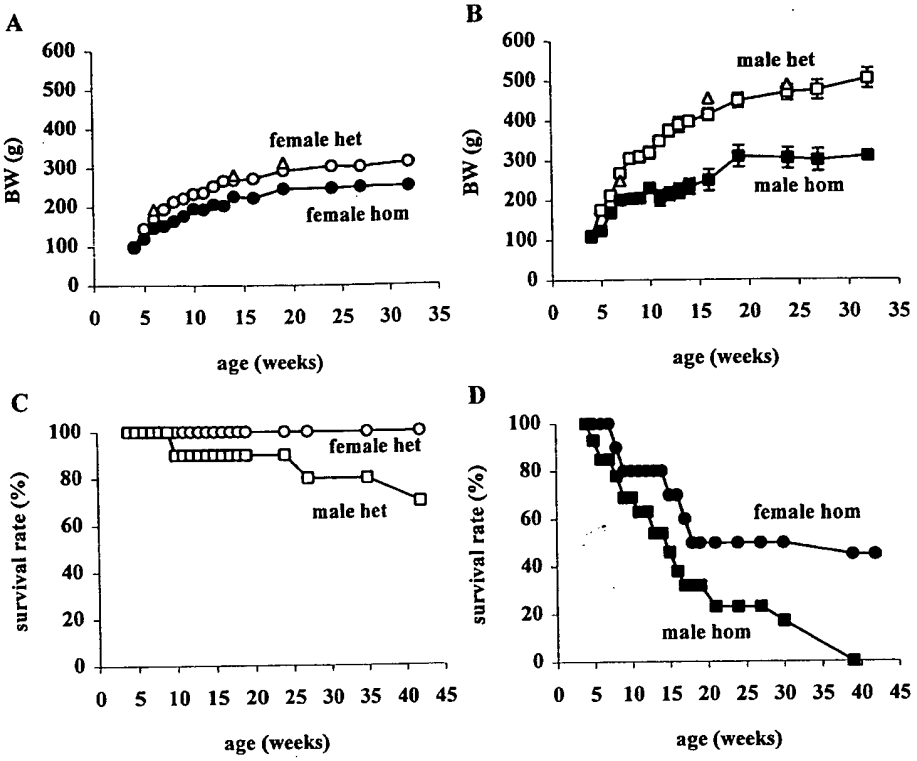

Fig. 2. Development of body weight in female (*A*) and male (*B*) heterozygous (het) and homozygous (hom) transgenic rats. Triangles, values for age-matched SD rats. Values are means ± SD; n = 10/group. $P < 0.005$, or 0.0005 beyond 7 wk of age, for comparison between heterozygous and homozygous rats. *C* and *D*: survival rates in TGR(mREN2)27 rats.

ity to study the role of the renin gene in sex-specific differences of hypertension, a phenomenon that is also observed in other rat strains (21) and in humans (13). Interestingly, gender-dependent phenotypic differences were also observed for certain components of the plasma RAS. Higher plasma angiotensinogen, observed in both transgenic and control males, may be attributed to androgens, which have been shown to stimulate hepatic angiotensinogen gene transcription (11). However, prorenin, ANG I, and ANG II were higher only in transgenic males compared with females, pointing to a transgene-related mechanism for this sex difference. *Ren*-2 gene expression is positively modulated by androgens (82). Furthermore, treatment of female DBA/2 mice with testosterone leads to an enhanced transcriptional activity of the *Ren*-2 gene in the submandibular gland (87), whereas treatment of young female TGR(mREN2)27 rats with testosterone has been shown to increase blood pressure to the levels of males (2). These findings indicate that androgens influence blood pressure by affecting *Ren*-2 gene expression. Peters et al. (59) demonstrated that most of the prorenin, as well as a substantial part of renin, in the circulation of TGR(mREN2)27 rats is of transgene origin. Therefore, androgen-stimulated *Ren*-2 gene expression may account for higher prorenin levels in male transgenic rats. Relatively higher levels of ANG I and ANG II in male than in female TGR(mREN2)27 rats may result from enhanced kinetics between rat angiotensinogen and mouse renin (81).

Tissue-Specific Phenotypic Characteristics

The transgene is expressed in a large number of tissues, with the highest expression found in adrenal gland, thymus, brain, and gastrointestinal and urogenital tracts, whereas *Ren*-2 gene expression in the kidney is low (51, 92). Comparison of the *Ren*-2 gene expression pattern between DBA/2 mice and transgenic rats demonstrates maintenance of correct tissue specificity except for the submandibular gland, where no *Ren*-2 transcripts were detectable in the transgenic rat despite high expression in the mouse (51, 92). This is most likely due to the presence of trans-acting factors in the murine submandibular gland, because the transgene includes 5′-flanking sequence elements that have been shown to confer tissue-specific expression of a reporter gene in mice lacking an endogenous *Ren*-2 gene in their genome (72). Because *Ren*-2 gene expression occurs in tissues in which low renin expression is observed in normal rats, overexpression of the transgene, rather than ectopic expression, may lead to activation or interference with preexistent local RAS. This renders TGR(mREN2)27 rats a suitable model for studying the regulation of renin gene expression and its role in cardiovascular disease at these sites.

Adrenal gland. The adrenal gland is the major site of *Ren*-2 gene expression in the transgenic rat (3, 51, 91). Overexpression of the transgene is accompanied by an increased mineralocorticoid excretion in young animals during the phase of hypertension development (66). Because the circulating RAS, a major stimulator of

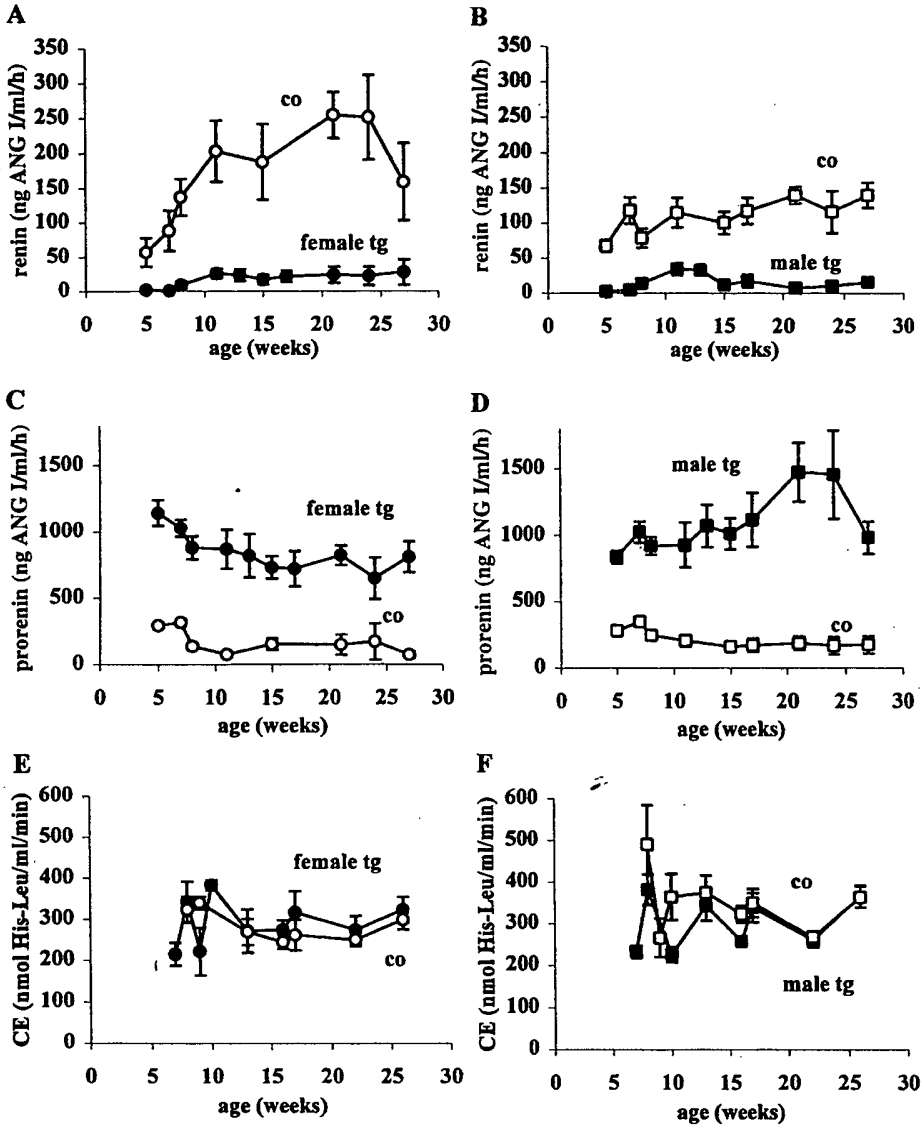

Fig. 3. Plasma renin and prorenin in female (*A*, *C*) and male (*B*, *D*) heterozygous transgenic (tg) and control rats (co) from 5 to 27 wk of age determined by indirect radioimmunoassay at pH 7.2 and after trypsin activation, respectively. Plasma converting enzyme activity (CE) in female (*E*) and male (*F*) heterozygous TGR(mREN2)27 rats. Values are means ± SD; $n = 10$/group. $P <$ 0.005 or 0.0005 at all time points for comparison of renin and prorenin between transgenic and control animals.

aldosterone release, is suppressed, an activated adrenal RAS might be responsible for the enhanced steroid metabolism. Indeed, in addition to adrenocorticotropic hormone and potassium, ANG II functions as a major regulator of steroid synthesis in the adrenal cortex, and it is increased in the adrenal gland of TGR(mREN2)27 rats (10, 39, 50). Furthermore, renin has been localized in mitochondrial dense bodies where steroidogenesis takes place, suggesting that locally produced ANG II may mediate the stimulation of basal aldosterone synthetase expression in the adrenal cortex of TGR(mREN2)27 rats (63, 67). Sodium restriction in transgenic rats was shown to selectively stimulate adrenal renin and to increase aldosterone synthesis, whereas the rise in plasma and renal renin was blunted compared with SD rats (64). This stimulation was abolished by AT_1 receptor blockade, indicating that mineralocorticoid synthesis in the transgenic rat is mediated by the AT_1 recptor (85). Renin and prorenin are secreted by isolated glomerulosa cells and are mainly of transgene origin, as demonstrated by immunoprecipitation with a mouse renin-specific antibody (59). The adrenal gland also appears to be the major source for circulating prorenin, because adrenalectomy caused a sustained decrease in plasma prorenin (3, 80). Moreover, ANG II has been demonstrated to stimulate secretion of renin, but not prorenin, in vitro. This implies different regulatory mechanisms for renin and prorenin secretion and indicates that adrenal renin is not subject to a negative feedback control by ANG II (59). The observation that the adrenal RAS is activated suggests that increased sodium and water retention due to oversecretion of mineralocorticoids may be responsible for hypertension in TGR(mREN2)27 rats. However, treatment with an aldosterone antagonist does not reduce or prevent hypertension, providing evidence that mineralocorticoids are not the major pathogenetic factor in the development of hypertension in TGR(mREN2)27 rats

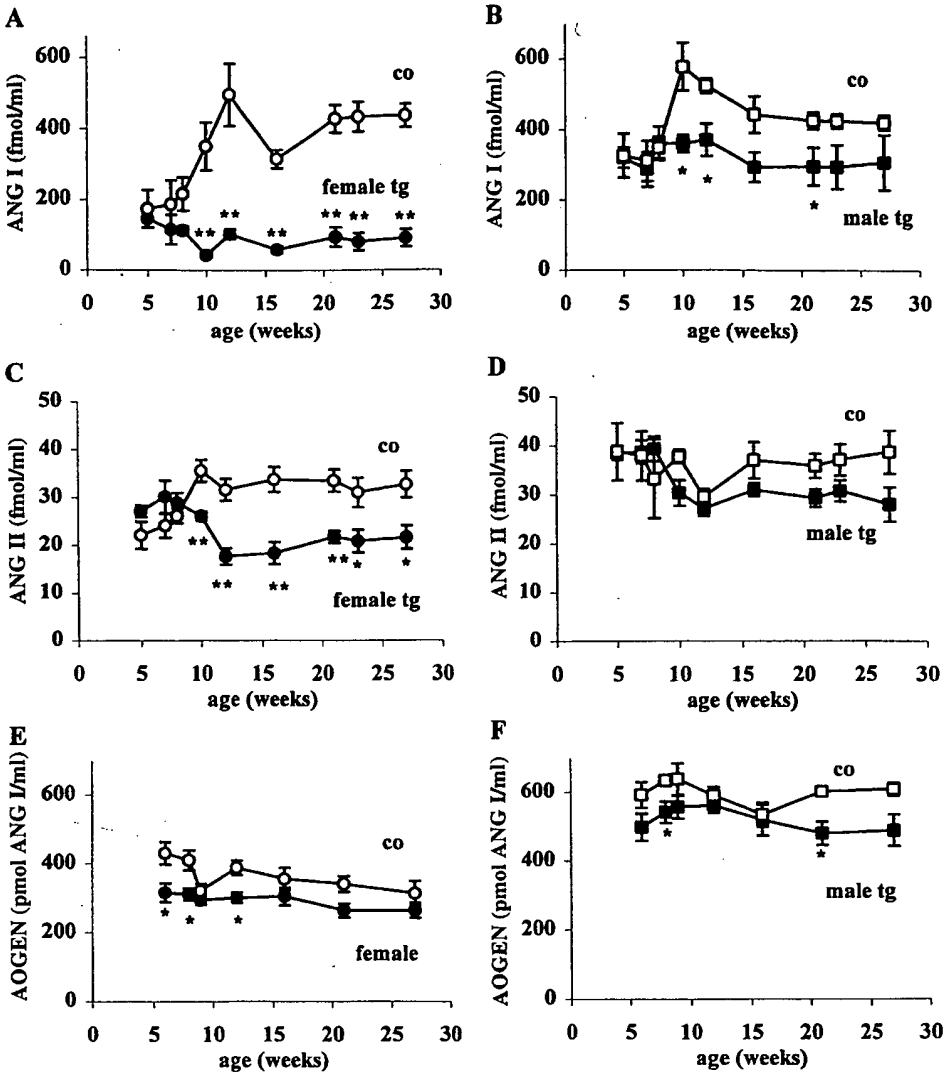

Fig. 4. Plasma angiotensin I (ANG I), angiotensin II (ANG II), and angiotensinogen (AOGEN) in female (*A*, *C*, and *E*) and male (*B*, *D*, and *F*) heterozygous transgenic (tg) and control (co) rats. Values are means ± SD; n = 10/group. $^{*}P < 0.05$ and $^{**}P < 0.005$ vs. control.

(66). This raises the possibility that other tissues that overexpress renin, such as heart, vasculature, or brain, may contribute to the hypertensive phenotype by yet undefined mechanisms.

Heart and vasculature. Growing evidence supports the existence of a self-contained cardiac angiotensin system that may be involved in adaptive processes associated with cardiac hypertrophy (45). Modulation of the expression of components of the RAS, such as converting-enzyme, angiotensinogen, and ANG II receptors, has been demonstrated in several models of cardiac overload (45, 76). Cardiac renin expression has also been demonstrated, albeit at very low levels (18, 58). Isolated cardiomyocytes have been shown to release ANG II in response to mechanical stretch (65). Furthermore, ANG II has been demonstrated to exert growth-promoting effects on isolated chicken myocytes (5) and to induce expression of protooncogenes in smooth muscle cells in vitro (53). Expression of protooncogenes is thought to be one mechanism by which reprogramming of cardiac gene expression in response to chronic volume overload is achieved (31, 68). Recruitment of the cardiac RAS may thus play an important role in pathogenesis of cardiac hypertrophy.

In the transgenic rats, the *Ren*-2 gene is also expressed in the heart and the vasculature, and this expression is accompanied by increased tissue concentrations of ANG II (10, 39, 92). Furthermore, Hilgers et al. (26) demonstrated an enhanced synthesis and release of ANG I and ANG II in isolated perfused hindlimbs of nephrectomized transgenic rats, an experimental setting where blood-borne renin cannot contribute to angiotensin biosynthesis. Transgenic rats develop pathomorphological changes of the cardiovascular system such as myocardial hypertrophy, which is accompanied by an increase in connective tissue and perivascular fibrosis (3). Vascular alterations include increased media thickness and hypertrophied smooth muscle cells of the aorta, as well as an increased media-to-lumen ratio without vascular growth of the mesenteric vasculature, which is consistent with remodeling (3, 79). In addition, functional and biochemical changes similar to those observed in human heart failure have been described in TGR(mREN2)27 rats, including down-

regulation of β-adrenergic receptors accompanied by a reduced positive inotropic response to isoproterenol (9, 78), as well as an increased expression of the gap-function protein, Connexin 40, the stimulation of which is thought to be a compensatory mechanism to increase conduction velocity (7). Moreover, endothelium-dependent contraction in response to the L-arginine analogue nitro-L-arginine methyl ester (L-NAME) in coronary arteries of transgenic rats is decreased, suggesting a reduced basal endothelial-derived nitric oxide release (83).

The question as to whether these changes are solely due to the elevated blood pressure, or whether local activation of the RAS is an independent factor contributing to the cardiovascular pathology in this model, remains uncertain. Enhanced local formation of ANG II, however, is consistent with an activated cardiac and vascular RAS, supporting the notion of its pathogenetic relevance in the transgenic rat. Cardiac dysfunction in TGR(mREN2)27 rats is more pronounced than in SHR, which exhibit a similar hemodynamic phenotype with regard to onset and degree of hypertension (57). Moreover, specific inhibitors of the RAS, such as converting-enzyme inhibitors or ANG II receptor antagonists, are capable of inducing regression of heart hypertrophy, even at low doses that only cause a slight reduction in blood pressure, whereas direct vasodilators fail to exhibit similar cardioprotective effects despite efficient blood pressure reduction (4, 8, 39).

Kidney. Despite the presence of an additional renin gene in the genome of TGR(mREN2)27 rats, renin activity in the kidney is markedly reduced (51). Whereas suppression of the endogenous rat renin gene is similar in hetero- and homozygous transgenic rats, *Ren*-2 gene expression is higher in homozygous animals and, therefore, transgene dose dependent (39). Interestingly, renal ANG II content is reduced in heterozygous animals compared with controls, whereas in homozygous animals it is unchanged or even increased, correlating with renal *Ren*-2 gene expression (10, 39). Thus renal ANG II, which appears to be inappropiately high at least in homozygous transgenic animals, may result from enhanced kinetics between mouse renin and rat renal angiotensinogen (81). Because intrarenal ANG II has been shown to exert a negative regulatory influence on renin expression (32), locally generated ANG II in the transgenic rat may participate in the suppression of the endogenous renin gene independently of blood pressure.

The role of the kidney in the development and maintenance of hypertension is well established, and several lines of evidence indicate that an intrarenal RAS is an important regulator of renal hemodynamics and function (42). TGR(mREN2)27 rats exhibit functional and structural changes of the kidney that are commonly observed in hypertension, such as a shift of the pressure-natriuresis relationship toward higher arterial pressure, reduced glomerular filtration rate, and albuminuria. The albuminuria appears as early as 8 wk of age and is associated with glomerulosclerosis (3, 22, 75). ANG II is a powerful modulator of sodium reabsorption and has recently been shown to upregulate proximal tubule expression of the AT_1 receptor (12). Therefore, elevated tubular sodium reabsorption that accompanies the resetting of the pressure-natriuresis response toward higher levels of renal perfusion pressure may be attributed to locally generated ANG II in the kidney in TGR(mREN2)27 rats (22). In addition, a study on the tubuloglomerular feedback mechanism suggests an increased responsiveness in the transgenic rat, which was attenuated to a greater extent after AT_1 receptor blockade than after mechanical reduction of renal perfusion pressure (46). This observation indicates that the increased tubuloglomerular feedback responsiveness is ANG II dependent.

ANG II receptor blockade, but not the combined regimen of reserpine, hydrochlorothiazide, and hydralazine, has been shown to limit glomerular injury in rats with reduced renal mass, implying that remnant glomerular function and structure depend on reduction of ANG II activity (38). Similarly, antihypertensive treatment with inhibitors of the RAS, but not hydralazine, is capable of halting the progression of glomerulosclerosis in TGR(mREN2)27 rats (8, 39), suggesting that these structural alterations may not solely be hypertension related, but primary events caused by interference or activation of the renal RAS because of the presence of the transgene.

Central and peripheral nervous system. Expression of the transgene in the brain of TGR(mREN2)27 rats is already present in newborn animals and appears to be developmentally regulated. In the hypothalamus, *Ren*-2 gene expression persists in adult animals, whereas it is downregulated in the medulla oblongata (92). This is accompanied by a marked elevation of ANG II in the brain of transgenic rats, indicating the presence of an activated RAS and suggesting a role for the central nervous system in the pathogenesis of hypertension in this strain (10, 69). In normal rats, angiotensinogen, the only known substrate for renin, is highly expressed in glial cells (60). Likewise, converting-enzyme and ANG II receptors have been readily demonstrated, whereas renin, the cellular localization of which is unknown, is minutely expressed within the central nervous system (1, 17).

ANG II is an important modulator of the sympathetic nervous system and is involved in the regulation of thirst and the release of vasopressin (60). Although an enhanced interaction between vascular ANG II and the sympathetic nervous system appears to be absent in TGR(mREN2)27 rats (27), central modulation of sympathetic tone may be one of the mechanisms by which the RAS in the brain participates in the hypertensive process. Interestingly, it has been demonstrated that the hypertensive effect, as well as the central vasopressin release in response to centrally administered ANG II in transgenic rats, is absent (48). Furthermore, the central distribution of vasopressin has been shown to be altered in TGR(mREN2)27 rats, suggesting an interaction between the central RAS and the vasopressin axis (48). Moriguchi et al. (48) demonstrated that intracerebrovascular administration of an ANG II-

specific antibody led to a decrease, whereas application of an ANG-(1—7) antibody led to an increase, in blood pressure in TGR(mREN2)27 but not in SD rats, suggesting opposing actions of ANG II and ANG-(1—7) in the brain of transgenic rats and supporting the contention of central ANG II in the pathogenesis of hypertension in the transgenic rat (49). Whether altered circadian rhythms of blood pressure may be related to overexpression of the transgene in the brain awaits further investigation (41).

MECHANISMS OF HYPERTENSION IN TGR(mREN2)27

TGR(mREN2)27 rats develop fulminant hypertension from the presence of an additional renin gene in their genome. Several lines of evidence indicate that the hypertensive phenotype is indeed determined by the transgene and not due to an insertional mutation. First, all transgenic founder animals were hypertensive irrespective of the transgene insertion site within the genome. Second, hypertension cosegregates with the transgene, and, third, hypertensive transgenic rats respond with great sensitivity to antihypertensive treatment with converting-enyzme inhibitors or the ANG II receptor antagonist, indicating that hypertension is ANG II dependent (4, 8, 39, 51).

One of the most striking features of TGR(mREN2)27 is the suppression of the circulating renin, which appears to exclude the plasma RAS as the cause for the hypertensive phenotype (51). High expression of the transgene is associated not only with an increased renin activity but also with an enhanced local formation of ANG II, indicating that the transgene is translated into a functional protein (10, 26, 39, 51). Furthermore, tissue-specific differential modulation of gene expression of RAS components suggests the presence of different regulatory and functional properties of the RAS at these sites (39). Local formation of ANG II in organs involved in cardiovascular homeostasis supports the idea of a causal role of extrarenal RAS in the pathogenesis of hypertension and structural alterations, such as cardiovascular hypertrophy and glomerulosclerosis.

The transgenic rat, therefore, demonstrates in a very intriguing way that the RAS can be causally involved in the hypertensive process, even in absence of elevated circulating renin. This represents probably one of the most convincing pieces of evidence for a physiological significance of tissue-resident RAS and suggests that they may be involved in the pathogenesis of other forms of hypertension associated with normal or low plasma renin. This contention is in agreement with the commonly observed lack of correlation between the antihypertensive effect of converting-enzyme inhibitors and plasma renin or converting-enzyme levels before and after treatment (84, 86). The findings in the transgenic rat confirm the hypothesis that plasma levels of the components of the RAS may not always be an appropriate indicator for the activity of the system, because they do not allow any conclusions as to the activity of local RAS.

Another characteristic feature of the transgenic rat is the elevated prorenin that is mainly of mouse origin and, therefore, transgene derived (59). The physiological significance of circulating prorenin is still uncertain, and the question whether it is intrinsically active, or undergoes local activation in vivo, is controversially discussed (54). Exogenously applied prorenin is not activated in the circulation of SHR and monkeys but appears to be sequestrated and activated by the kidney without affecting blood pressure (29, 34). Similarly, it has been suggested that prorenin can be sequestrated from the coronary circulation in humans (73). The finding of prorenin-converting enzymes in rat aorta and kidney, as well as in human neutrophil cells (16, 77), has led to the hypothesis that circulating prorenin may be taken up locally and activated by lysosomal proteases or an acid pH milieu. Subsequent intracellular generation of ANG II may then influence local perfusion or exert trophic effects on the vascular wall. Interestingly, a recently established new transgenic rat strain, which harbors the *Ren*-2 gene fused to a liver-specific promoter, exhibits high plasma prorenin because of hepatic secretion and develops hypertension as well as cardiac hypertrophy (6). Because active *Ren*-2 gene-derived renin was detectable, this model suggests that extrarenal prorenin can be activated in vivo, leading to hypertension (6). However, whether high circulating prorenin in TGR(mREN2)27 rats merely represents an epiphenomenon or contributes to the cardiovascular phenotype requires further detailed investigation.

CONCLUSION AND OUTLOOK

In summary, TGR(mREN2)27 represents the first rat model of hypertension in which the genetic defect is precisely defined. Fulminant hypertension in the presence of suppressed plasma RAS and high renin expression in extrarenal tissues suggests that activated extrarenal RAS might be responsible for the hypertensive phenotype. Regulated expression of the transgene in concert with *cis*- and *trans*-regulatory factors appears to have tissue-specific effects on preexisting local RAS. The *Ren*-2 gene gives rise to a functional protein that leads to increased local formation of ANG II, which then acts on target tissues in a paracrine or autocrine manner. This local ANG II formation may be a key factor for the changes in cardiovascular morphology that appear to be partially independent from blood pressure.

The importance of ANG II in the regulation of blood pressure, fluid, and electrolyte balance, as well as cardiovascular growth, is well recognized. TGR(mREN2)27 rats overexpress renin in a number of extrarenal tissues that are not obviously linked with cardiovascular function, such as thymus, and gastrointestinal and urogenital tracts. This provides the unique opportunity to investigate the functional role of ANG II in these tissues. Among the more recent evidence for multiple and yet undefined functions of ANG II is the intricate developmental and tissue-specific regulation of the expression of ANG II receptors, notably the AT_2 recep-

tor, which is highly expressed in embryo and brain (33), as well as the existence of at least two converting-enzyme isoforms in testis and intestine, respectively, in addition to the endothelial isoform (14, 36). To explore such functions, the transgenic gain-of-function model should be complemented with loss-of-function models using homologous recombination, as recently demonstrated for several RAS components (74). Targeted disruption of the converting-enzyme gene in mice is associated with a slight reduction in blood pressure and male infertility, providing direct evidence for a role of angiotensin-converting enzyme in male gonadal function (35). Two reports on the homozygous deletion of the AT_2 receptor gene in mice describe changes in exploratory behavior as well as neurophysiological parameters, such as body temperature and algesia (25, 30). These findings further illustrate the wide spectrum of the functional properties of ANG II. In addition, mutant mice lacking the AT_2 receptor show an increased pressure response to ANG II, suggesting mutually counteracting hemodynamic effects between the AT_1 and the AT_2 receptors (25, 30).

Although TGR(mREN2)27 rats provide a large amount of information on the role of the RAS in cardiovascular disease, they cannot be considered as a genuine model of human hypertension, which is polygenic in nature. They do provide, however, a sophisticated approach to analyze phenotypic consequences of defined genetic alteration in an in vivo system. Despite the fact that the exact mechanisms underlying the phenotype are still uncertain, the transgenic rat paradigmatically demonstrates that alterations within extrarenal RAS may result in a phenotype characterized by hypertension and pathological changes within the cardiovascular system. Therefore one may speculate that similar alterations in extrarenal renin under yet undefined physiopathological circumstances may also be of relevance for some forms of human hypertension. Enhancing our knowledge regarding the transgenic rat may ultimately lead to a better understanding of the mechanisms by which the RAS contributes to the pathogenesis of hypertension, providing the basis for future studies in humans.

We thank Claire Bayer for expert technical assistance; Dr. Jürgen Bachmann for blood pressure measurements; and Dr. Klaus Lindpaintner, Dr. Speranza Rubattu, and Dr. Chris E. Talsness for helpful discussions.

This work was supported by Deutsche Forschungsgemeinschaft; National Heart, Lung, and Blood Institute; the European Community (Trans-Gen-Eur); and Bristol-Meyers-Squibb (Princeton, NJ).

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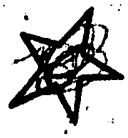

ADVANCES IN PHYSIOLOGY EDUCATION
See Part 3, S1–S93

JUNE 1996
Volume 270/Number 6
Part 1 of Three Parts
ISSN 0002-9513

American Journal of Physiology

PUBLISHED BY
THE AMERICAN PHYSIOLOGICAL SOCIETY

Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11153–11157, November 1994
Applied Biological Sciences

Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection

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Communicated by George E. Seidel, Jr., June 23, 1994

ABSTRACT **The serious shortage of human organs available for transplantation has engendered a heightened interest in the use of animal organs (xenografts) for transplantation. However, the major barrier to successful discordant xenogeneic organ transplantation is the phenomenon of hyperacute rejection. Hyperacute rejection results from the deposition of high-titer preformed antibodies that activate serum complement on the luminal surface of the vascular endothelium, leading to vessel occlusion and graft failure within minutes to hours. Although endogenous membrane-associated complement inhibitors normally protect endothelial cells from autologous complement, they are species restricted and thus confer limited resistance to activated xenogeneic complement. To address the pathogenesis of hyperacute rejection in xenotransplantation, transgenic mice and a transgenic pig were engineered to express the human terminal complement inhibitor hCD59. High-level cell surface expression of hCD59 was achieved in a variety of murine and porcine cell types, most importantly on both large vessel and capillary endothelium. hCD59-expressing porcine cells were significantly resistant to challenge with high-titer anti-porcine antibody and human complement. These experiments demonstrate a strategy for developing a pig-to-primate xenogeneic transplantation model to test whether the expression of a human complement inhibitor in transgenic pigs could render xenogeneic organs resistant to hyperacute rejection.**

The lack of effective therapies aimed at eliminating antibody- and complement-mediated hyperacute rejection presents a major barrier to the successful transplantation of discordant animal organs into human recipients (1–6) and has precluded the development of animal models aimed at evaluating the *in vivo* cellular immune response to discordant xenografts. Old World primates, including humans, have high levels of preexisting circulating natural antibodies that predominantly recognize carbohydrate determinants expressed on the surface of xenogeneic cells from discordant species (2–6). Recent evidence indicates that most of these antibodies react with the carbohydrate epitope, Gal(α1-3)Gal (7), an epitope absent from Old World primates because of a lack of the functional α-1,3-galactosyltransferase enzyme (8). Therefore, after transplantation of a vascularized xenogeneic donor organ into a primate recipient, the massive inflammatory response that ensues from natural antibody activation of the classical complement cascade leads to activation and destruction of the vascular endothelial cells and ultimately of the donor organ within minutes to hours after revascularization (2–6). Endogenously expressed membrane-associated complement regulatory proteins normally protect endothelial cells from autologous complement. However, the activity of these complement inhibitors is species restricted. This property makes them relatively ineffective at inhibiting xenogeneic serum complement (9, 10). The demonstration that a human complement inhibitor could protect a xenogeneic cell from human complement-mediated lysis showed that it was possible to inhibit human anti-porcine hyperacute rejection in *in vitro* models (11).

The strategy used to address the pathogenesis of hyperacute rejection in the porcine-to-primate xenotransplantation model was to produce transgenic swine expressing high levels of the human terminal complement inhibitor hCD59. hCD59 is an 18- to 20-kDa glycosyl-phosphatidylinositol-anchored cell surface glycoprotein that is expressed in a variety of tissues of both hematopoietic and nonhematopoietic lineage and functions to inhibit formation of the membrane attack complex by binding to membrane C5b-8 and C5b-9 (9, 10). Stable expression of hCD59 on xenogeneic cells *in vitro* protected the cells from human complement-mediated cell lysis (12–14) and the level of protection was directly proportional to the number of molecules of hCD59 expressed on the surface of the xenogeneic cell (14). Importantly, hCD59-expressing porcine aortic endothelial cells were resistant not only to cell lysis but also to complement-mediated formation of a procoagulant surface when challenged with either human or baboon serum (15). Taken together, these results indicated that high-level expression of hCD59 could provide porcine tissue with significant protection from human serum complement in a xenotransplantation setting. Therefore, hCD59 was chosen as a candidate molecule for production of transgenic swine resistant to human complement. In this report, we demonstrate the successful production of a transgenic pig expressing high levels of hCD59 that protect the pig cells from human complement-mediated cell lysis.

MATERIALS AND METHODS

***H2K^b*–hCD59 DNA Construct, Purification, and Microinjection.** A hCD59 cDNA was directionally cloned into exon 1 of the murine *H2K^b*-gene 12 nucleotides downstream of the transcriptional start site. Briefly, the hCD59 cDNA fragment was excised from a hCD59-pcDNAI-Amp (pcDNAI-Amp; Invitrogen) expression plasmid by digestion with *Hin*dIII, followed by enzymatically filling in the 5′ 4-nucleotide overhang with T4 DNA polymerase and dNTPs. Subsequently,

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Abbreviations: MHC, major histocompatibility complex; PBMCs, peripheral blood mononuclear cells; mAb, monoclonal antibody; PHA, phytohemagglutinin; FITC, fluorescein isothiocyanate; hrTNF-α, human recombinant tumor necrosis factor α; IFN, interferon.

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the DNA was digested with *Not* I at the 3′ end of the multiple cloning site of the vector to yield a 452-bp cDNA fragment. The 9.0-kbp *Eco*RI $H2K^b$ genomic restriction fragment (16) cloned into pGEM7Z (Promega) was digested with *Nru* I and *Not* I, resulting in the removal of 51 nucleotides from the $H2K^b$ gene including the ATG start codon. The hCD59 cDNA was then directionally ligated into the $H2K^b$ gene in the pGEM7Z vector.

Purification of the $H2K^b$–hCD59 DNA for embryo injection was accomplished by digesting the plasmid with *Xho* I to remove the vector sequences followed by agarose gel electrophoresis, electroelution, and Elutip purification (Schleicher & Schull). Transgenic mice were produced by pronuclear microinjection of murine ova as described (17). Ten of 60 offspring were identified as transgenic founder animals by DNA slot blot hybridization (18) (data not shown). Transgenic swine were generated by porcine embryo injection (19). A total of 18 piglets were analyzed by DNA slot blot analysis of genomic DNA (18). One founder animal, $H2K^b$–hCD59 153-2, contained 10–20 copies of the $H2K^b$–hCD59 DNA. Two additional founder animals, $H2K^b$–hCD59 152-1 and $H2K^b$–hCD59 152-2, contained ≈1 copy of the $H2K^b$–hCD59 DNA and exhibited no expression or very low and inconsistent levels of expression in peripheral blood mononuclear cells (PBMCs) (data not shown). These animals were not analyzed further.

Cell Culture, Immunofluorescence, and Immunohistochemistry. PBMCs from transgenic and negative littermate control pigs were purified from whole blood by Ficoll gradient centrifugation (ref. 20, pp. 7.1.1–7.1.2). Adherent monocytic mononuclear cells were cultured in Dulbecco's modified Eagle's medium/15% fetal bovine serum. PBMCs from transgenic mice and negative littermate control animals were purified from whole blood by ACK lysis (Biofluids, Rockville, MD). Indirect immunofluorescence of porcine PBMCs was performed with the anti-hCD59 mouse monoclonal antibody (mAb) MEM-43 (Biodesign International, Kennebunkport, ME) and with the anti-swine leukocyte antigen (SLA) class I mAb PT85A (VMRD, Pullman, WA). Indirect immunofluorescence of murine PBMCs was performed with polyclonal antisera specific for hCD59 (P. Sims, Blood Research Institute, Milwaukee). Goat anti-rabbit IgG (polyclonal sera; Zymed) or goat anti-mouse IgG (monoclonal sera; Zymed) fluorescein isothiocyanate (FITC)-conjugated antisera were used to detect specific antibody binding to the cell surface. Cell surface expression was then measured by flow cytometry on a Becton Dickinson FACSort.

The cytokine inducibility of $H2K^b$–hCD59 and the endogenous porcine SLA class I molecule was tested on adherent peripheral blood monocytes. Briefly, porcine cytokine-conditioned medium supernatants were produced from control pig PBMCs. PBMCs harvested from a control pig were stimulated with phytohemagglutinin (PHA; 5 μg/ml) for 48 h. PHA-conditioned media were collected and treated with 10 mM methyl α-mannoside and filter sterilized. Human recombinant tumor necrosis factor α (hrTNF-α; Collaborative Biomedical Products, Bedford, MA) was used at 500 units/ml. Adherent peripheral blood monocytes were then treated with medium alone, 50% PHA-conditioned medium (diluted 1:1 with complete medium), 50% PHA-conditioned medium/hrTNF-α, or hrTNF-α for 24 h. Cytokine-induced expression of hCD59 and SLA class I was detected by immunofluorescence and fluorescence-activated cell sorter analysis as described above.

Immunohistochemistry was performed on fresh frozen sections embedded in Tissue-Tek OCT compound (Miles). Tissue sections (5–10 μm) were processed as described (ref. 20, pp. 5.8.1–5.8.2). Sections that were double stained were processed simultaneously with the mouse anti-hCD59 mAb, MEM-43 (20 μg/ml), and the anti-type IV collagen rabbit polyclonal antiserum (21) (1:50 dilution). Fluorochrome-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antisera were used to detect specific antibody interactions with the hCD59 antigen (goat anti-mouse rhodamine; AMAC, Westbrook, ME) and type IV collagen antigen (goat anti-rabbit FITC; Zymed).

Complement-Mediated Dye Release Assays. PBMCs or peripheral blood adherent cells were labeled with the intracellular dye Calcein AM (Molecular Probes). The cells were subsequently incubated with anti-porcine blood cell IgG (2 mg/ml) (Intercell Technologies, Hopewell, NJ) followed by incubation in increasing concentrations of human whole serum (Sigma) at 37°C for 30 min. Dye released from the cells was determined by flow cytometry on a Becton Dickinson FACSort. The C5b-9-specific dye release was calculated as percentage of total, correcting for nonspecific dye release and background fluorescence measured on identically matched controls without the addition of serum. Antibody blocking experiments were performed by the complement-mediated dye release assay as described above with the following exceptions. The cells were incubated in 20% C8-deficient serum (C8d; Quidel, San Diego) at 37°C for 30 min after anti-porcine blood cell antibody activation. The cells were then incubated with hCD59 polyclonal antiserum (100 μg/ml) or anti-SLA class I antiserum PT85A (100 μg/ml). Purified human C8 (Quidel) and C9 (Quidel) complement components were then added in increasing concentrations and incubated at 37°C for 30 min. Dye released from the cells was detected by flow cytometry on a Becton Dickinson FACSort as described above.

RESULTS

Transgenic Expression. To achieve expression of the transgene-encoded hCD59 we engineered a murine major histocompatibility complex (MHC) class I gene, $H2K^b$ (16), to control the expression of a hCD59 cDNA, $H2K^b$–hCD59 (Fig. 1). The MHC class I gene is ubiquitously expressed on most somatic cells and, most importantly, is a predominant endothelial cell surface antigen (22, 24). In addition, the MHC class I promoter contains cis-acting regulatory elements that bind cytokine-inducible trans-acting factors, resulting in upregulation of the class I gene upon stimulation with interferon (IFN)-α/β, IFN-γ, and TNF-α (22–25). A hCD59 cDNA was cloned into exon I of $H2K^b$ and results in a transcript that initiates at the $H2K^b$ transcriptional start site and proceeds through both the cDNA insert and the entire transcriptional unit of the $H2K^b$ gene. Translation initiates at the ATG codon of the inserted cDNA and terminates at the cDNA stop codon. The rest of the $H2K^b$ gene remains untranslated and functions only in RNA processing, providing the cDNA with a genomic structure that contains all the regulatory elements required for $H2K^b$ expression (22–25).

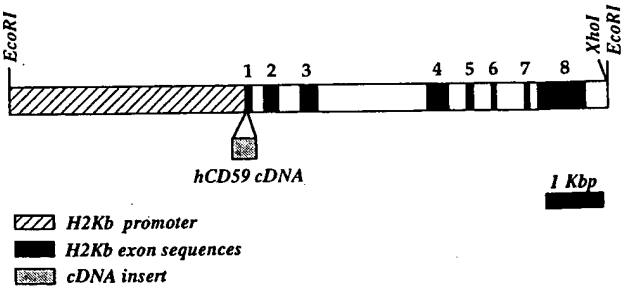

FIG. 1. $H2K^b$ genomic cassette. A linear representation of the hybrid gene construct detailing the exon–intron structure of $H2K^b$ and the insertion of the hCD59 cDNA into exon 1.

The efficacy of the *H2K*^{b}–hCD59 genomic expression construct in directing cell surface expression of hCD59 in various tissues was tested in transgenic mice and pigs. Initial analysis demonstrated that the *H2K*^{b}–hCD59 genomic construct directed the expression of hCD59 on the surface of PBMCs in several founder transgenic mice and transgenic pig 153-2 (Fig. 2 *A* and *B*, respectively). Importantly, expression of hCD59 on the surface of the porcine mononuclear cells paralleled that of SLA class I (Fig. 2*B*). The comparable expression of hCD59 to SLA class I indicated that the murine

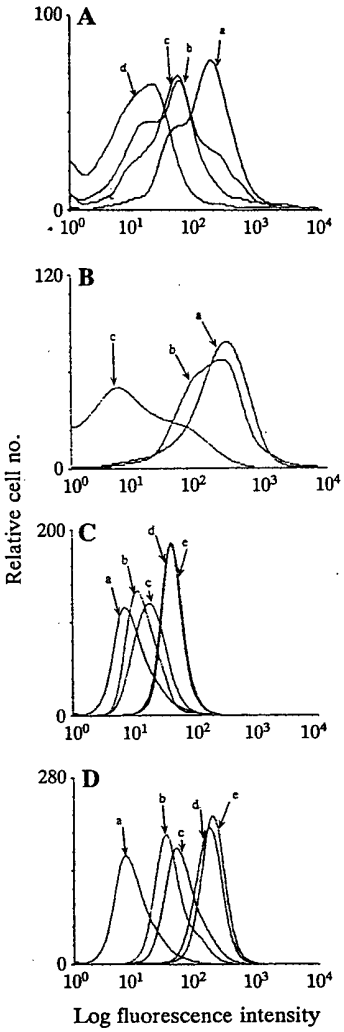

FIG. 2. Cell surface expression of hCD59 in transgenic mice and a transgenic pig. (*A*) Expression of hCD59 on murine PBMCs detected in transgenic mice *H2K*^{b}–CD59-11 (curve a), *H2K*^{b}–CD59-23 (curve b), *H2K*^{b}–CD59-21 (curve c), and a negative littermate control (curve d). (*B*) Cell surface expression of hCD59 and SLA class I detected on porcine PBMCs. Curve a, hCD59 expression in transgenic pig *H2K*^{b}–hCD59 153-2; curve b, SLA class I expression in transgenic pig *H2K*^{b}–hCD59 153-2; curve c, negative littermate control PBMCs incubated with the hCD59 mAb. (*C*) Cytokine-induced cell surface expression of hCD59 on cultured adherant PMBCs from pig *H2K*^{b}–hCD59 153-2; goat-anti-mouse FITC control antisera (curve a); hCD59 expression on uninduced cells (curve b); hrTNF-α (curve c); PHA conditioned medium (curve d); PHA conditioned medium + hrTNF-α (curve e). (*D*) Cytokine-induced cell surface expression of SLA class I on cultured adherent PBMCs from pig *H2K*^{b}–hCD59 153-2; goat-anti-mouse FITC control antiserum (curve a); uninduced cells (curve b); hrTNF-α (curve c); PHA conditioned medium (curve d); PHA conditioned medium + hrTNF-α (curve e).

H2K^b–hCD59 chimeric gene was constitutively regulated, similar to the endogenous porcine SLA class I molecules. To establish whether the *H2K*^{b}–hCD59 chimeric gene exhibited cytokine inducibility comparable to the endogenous SLA class I gene, we cultured adherent monocytic PBMCs. Interestingly, after prolonged culture, these monocytes had downregulated cell surface expression of both SLA class I as well as the hCD59 transgene-encoded protein (compare Fig. 2*B*, curve b, to Fig. 2*D*, curve b for class I and Fig. 2*B*, curve a, to Fig. 2*C*, curve b, for hCD59). Treatment of the transgenic porcine cells with PHA-induced cytokine-conditioned medium, with hrTNF-α, or with a combination of the treatments resulted in an increase in hCD59 expression (Fig. 2*C*) as well as an increase in SLA class I expression (Fig. 2*D*).

We next examined hCD59 expression on the endothelium of vascularized organs. Immunohistochemical analyses were performed on fresh-frozen tissue sections derived from hCD59 transgenic mice and pigs as well as from nontransgenic littermates. Phase-contrast micrographs illustrating the structure of mouse myocardium are shown in Fig. 3 *A* and *D*. Tissue sections from three founder mice were analyzed for hCD59 expression. Mouse hearts were incubated with anti-collagen type IV polyclonal antisera to detect basement membrane structures underlying the endocardium as well as intramyocardial capillary endothelia (21). Fig. 3 *B* and *E*, respectively, confirmed equivalent collagen staining in the negative littermate control and a representative hCD59 transgenic mouse, *H2K*^{b}–hCD59-8. In contrast, staining with a mAb specific for hCD59 revealed intense cell surface expression on endothelial cells in the heart of transgenic mouse *H2K*^{b}–hCD59-8 (Fig. 3*F*) and an absence of hCD59 expression in the negative littermate control (Fig. 3*C*). Fig. 3*F* dramatically highlights the expression of hCD59 on vascular structures and clearly shows high-level expression of hCD59 on the endocardium in the ventricular chamber. Abundant hCD59 was also detected on capillary vessels within the myocardium (Fig. 3*F*). All three founder transgenic mice analyzed revealed hCD59 staining on the endocardium and capillary endothelium. To evaluate vascularized structures in the transgenic pig without having to sacrifice the founder animal, tail sections were prepared and analyzed by immunohistochemistry as described for the mice. Phase-contrast micrographs illustrate the morphological structure of a tail artery from a negative control pig (Fig. 4*A*) and a tail artery

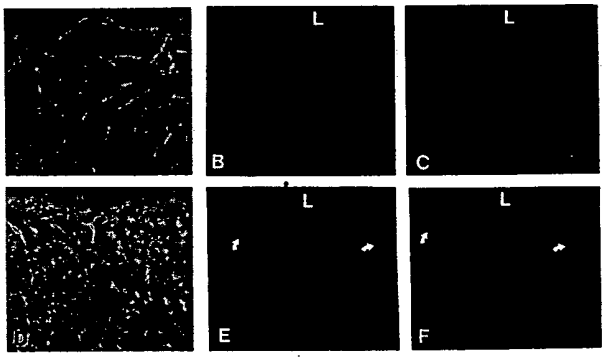

FIG. 3. Double-label immunofluorescence microscopy of hCD59 and type IV collagen on murine heart tissue from a *H2K*^{b}–hCD59 transgenic mouse and a negative littermate control. Phase-contrast micrographs of murine ventricular myocardium (*A* and *D*). L, lumen of the left ventricle lined by endothelial cells. (*B* and *E*) Immunofluorescence micrographs detecting type IV collagen (fluorescein) of the same myocardial sections illustrating basement membrane structures underlying the endocardium. Immunofluorescence micrographs (rhodamine) of the same myocardial sections detecting hCD59 in a negative littermate control (*C*) and *H2K*^{b}–hCD59-8 (*F*). (×400.) (Bar = 25 μm.)

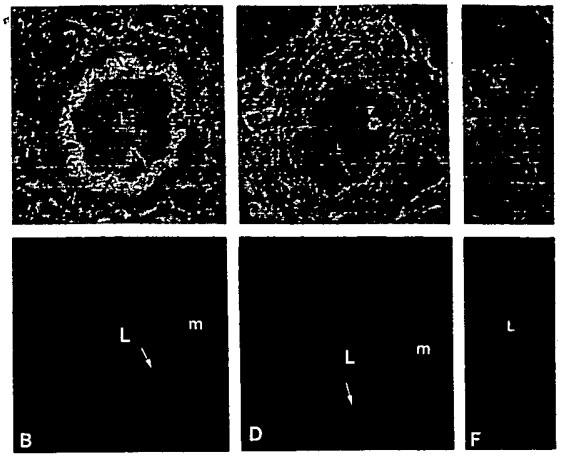

FIG. 4. Immunofluorescence microscopy of hCD59 on swine tail sections from pig *H2K*^{b}–hCD59 153-2 and a negative littermate control. (*A*) Phase-contrast micrograph of a dermal artery from the negative littermate illustrating the lumen (L), the endothelial cell layer (arrow), and the tunica media (m). (*B*) Immunofluorescence micrograph (rhodamine) of the same section pictured in *A*, illustrating the lumen, the endothelial cell layer, and the tunica media. (*C*) Phase-contrast micrograph of a dermal artery from pig *H2K*^{b}–hCD59 153-2, illustrating the lumen, the endothelial cell layer, and the tunica media. (*D*) Immunofluorescence micrograph (rhodamine) of the same section pictured in *C*, illustrating the lumen, the endothelial cell layer, and the medial smooth muscle cells (m). (*E*) Phase-contrast micrograph of a dermal microvessel from pig *H2K*^{b}–hCD59 153-2, illustrating the lumen and the vessel wall. (*F*) Immunofluorescence micrograph (rhodamine) of the same section pictured in *E*, illustrating the lumen, and an abundance of hCD59 expression. (×400.) (Bar = 25 μm.)

and small vessel from the transgenic founder pig 153-2 (Fig. 4 *C* and *E*, respectively). High-level hCD59 expression was observed on a variety of tissue and cell types, including fibroblasts, epithelial cells, vascular endothelial cells, and smooth muscle cells within the tail section of the transgenic pig (Fig. 4 *D* and *F*) but not in the negative littermate (Fig. 4*B*). Not all tissue in the transgenic pig tail section revealed hCD59 staining; however, tissues such as striated muscle are known to express very low levels of the class I antigen and therefore would not be expected to express the class I-regulated hCD59 transgene (24). These analyses confirmed that the *H2K*^{b}–hCD59 genomic construct directed expression of hCD59 to a variety of cells and tissues in transgenic pig 153-2 and, most importantly, to the surface of vascular endothelial cells.

Complement Resistance. To determine whether the high levels of transgene expression observed on the transgenic pig cells conferred significant protection from human complement-mediated attack, functional analyses were performed on hCD59-expressing porcine PBMCs collected from transgenic pig 153-2 and a nontransgenic littermate control. The data clearly demonstrated that hCD59-expressing porcine cells, but not cells from a nontransgenic littermate, significantly resisted human complement-mediated lysis (Fig. 5*A*). The percentage dye released from hCD59 protected cell was ≈5-fold less when compared with the amount of dye released from negative littermate control cells. To confirm that the protection observed in PBMCs was due specifically to hCD59 expression, antibody blocking experiments were performed. As shown in Fig. 5*B*, the anti-hCD59 polyclonal antisera blocked the hCD59-mediated protection, resulting in an increased susceptibility of the porcine cells to human complement-mediated cell lysis. In contrast, the control antibody had no effect.

To evaluate whether the degree of protection of porcine cells from human complement attack was a function of the

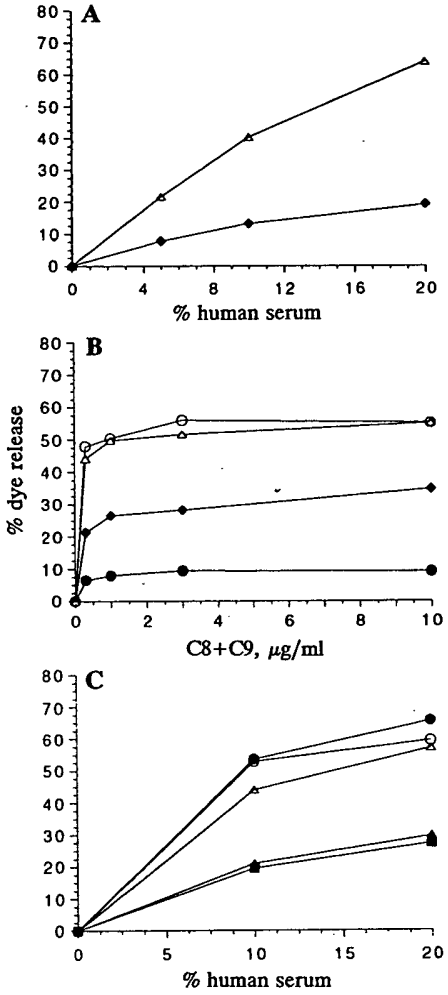

FIG. 5. Complement-mediated dye release assays on porcine PBMCs and cultured peripheral blood adherent cells. (*A*) Dye release assay performed on porcine PBMCs (◆), transgenic pig *H2K*^{b}–hCD59 153-2; (△), negative littermate control. (*B*) Dye release assay performed on PBMCs from transgenic pig *H2K*^{b}–hCD59 153-2 incubated in the presence of anti-hCD59 polyclonal antiserum (◆); control class I antibody PT85A (●); negative littermate control PBMCs incubated in the presence of anti-hCD59 polyclonal antiserum (△); control class I antibody PT85A (○). (*C*) Complement-mediated dye release assays on porcine peripheral blood adherent cells from pig *H2K*^{b}–hCD59 153-2; uninduced cells (△), PHA supernatants (■), PHA supernatants + hrTNF-α (▲), hrTNF-α (●), and control negative littermate cultured peripheral blood adherent cells (○).

level of hCD59 expressed on the cell surface, experiments were performed on the cultured monocyte lines derived from the *H2K*^{b}–hCD59 153-2 transgenic pig, which showed increased cell surface expression in response to cytokine treatments (see Fig. 2). Significantly, these monocytes demonstrated increased susceptibility to human complement-mediated lysis, consistent with the loss of hCD59 expression (Fig. 5*C*). As previously shown, culture of these cells in the presence of cytokines known to induce the MHC class I promoter—i.e., IFN-γ and TNF-α—upregulated hCD59 expression (Fig. 2*C*). Importantly, upregulating hCD59 expression restored their complement-resistant phenotype (Fig. 5*C*). These results confirm that the level of transgene expression correlates with cellular protection and also highlight the potential utility of the inducible *H2K*^{b} promoter in the setting of a cytokine-mediated inflammatory response.

DISCUSSION

Expression of human complement inhibitor hCD59 was established in transgenic mice and in a transgenic pig utilizing the murine MHC class I gene as a genomic expression cassette. The proteins encoded by the MHC class I genes from human (HLA), mouse (MHC), and swine (SLA) are expressed in most somatic cell types including the vascular endothelium (22, 24, 26). Therefore, a MHC class I promoter should direct high-level transgene expression in the endothelial cells of vascularized organs. The additional advantage to this genomic expression strategy is that the class I promoter has the capacity to upregulate hCD59 expression in response to the inflammatory cytokines IFN-γ and TNF-α (22, 24, 25).

We have approached the problem of complement-mediated hyperacute rejection during pig-to-primate xenotransplantation by engineering the xenogeneic donor tissue with human complement inhibitor hCD59. The analyses of hCD59 in *H2K^b*–hCD59 transgenic mice and transgenic pig 153-2 demonstrated that the *H2K^b*–hCD59 genomic construct regulated the expression of hCD59 in the context of a transgenic genome. Cell surface expression of hCD59 was detected in a variety of cells and tissues, including the vascular endothelium. The assays used to determine the protective effects of hCD59 expressed on the transgenic cells were performed with human whole serum, which contains serum complement components, as well as high-titer natural antibodies (W.L.F. and S.A.R., unpublished data). In addition, anti-porcine lymphocyte antiserum was used to enhance the activation of the classical complement pathway on the surface of the target cell. Our data demonstrated that the level of hCD59 expressed on the cell surface protected the xenogeneic cell even in the presence of additional complement-activating antibodies.

The utility of blocking complement as a method to prevent hyperacute rejection in pig-to-primate xenotransplantation was demonstrated by using cobra venom factor (CVF) and recombinant soluble complement receptor type 1 (sCR1) (refs. 27 and 28, respectively). A significant delay of complement-mediated hyperacute rejection in pig-to-primate heterotopic cardiac xenotransplantation was observed with the administration of CVF for two consecutive days before transplantation (27) or with a single intravenous bolus of sCR1 before xenograft reperfusion (28). The advantage of developing a transgenic donor animal expressing a human complement inhibitor is to provide the donor tissue with an endogenously expressed membrane-bound inhibitor and therefore does not rely on repeated administration of pharmacological agents.

The successful engineering of transgenic swine expressing a human complement inhibitor, and the demonstration that cells from these animals were significantly protected from human complement attack, suggests that this strategy may represent a useful component of an overall approach to discordant xenotransplantation. This transgenic approach will hopefully make porcine-to-primate transplantation models feasible that will allow the cellular aspects of discordant xenograft rejection to be evaluated. In addition, the production of porcine organs resistant to hyperacute rejection may open therapeutic windows for organ transplantation into humans, particularly when this technology is coupled with advances in cellular immunosuppressive regimens.

We thank Dr. Leonard Bell of Alexion for providing an intellectually stimulating environment and for insightful comments on the work. We would also like to thank Dr. Frank Gwazdauskas, Ed Guilmette, Stella Bianco-Caron, Stephanie DeCesare, and Adeline Tucker for excellent technical assistance.

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NOVEMBER 8, 1994
VOLUME 91
NUMBER 23

Proceedings
OF THE
National Academy of Sciences
OF THE UNITED STATES OF AMERICA

A Moloney MLV-Rat Somatotropin Fusion Gene Produc s Biologically Active Somatotropin in a Transgenic Pig

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Expression of a Moloney murine leukemia virus (MLV) rat somatotropin fusion gene was examined in a transgenic pig. The fusion gene was integrated in a single site within the genome in a tandem array with approximately eight copies per cell. The integrated MLV-rat somatotropin fusion gene produced high levels of circulating rat somatotropin and resulted in an elevation in the circulating levels of insulin-like growth factor I. Although there was no increase in the rate of growth of the transgenic animal during the rapid growth phase, several phenotypic changes were evident. Skeletal growth was markedly increased and fat deposition was reduced throughout the animal. Blood glucose levels were elevated without ketosis. Northern blot analyses of rat somatotropin RNA revealed that expression of the fusion gene was highest in the spleen, lung, intestine, lymph nodes, and bone marrow. These results show that the MLV promoter can be used to express high levels of biologically active rat somatotropin in transgenic swine. (Molecular Endocrinology 2: 277–283, 1988)

INTRODUCTION

The transgenic mouse model has provided fundamental information on gene expression and regulation (1). Although transgenic mice are convenient for laboratory studies, an important application of genetic manipulation in the future will be to develop the scientific and economic potential of transgenic livestock. In addition to the obvious economic benefits, transgenic domestic animals might provide useful models for studying hormonal abnormalities. Studies with transgenic mice have shown that dramatic phenotypic changes can be produced by introducing genes coding for growth-promoting proteins (2, 3). By analogy, expression of growth-promoting proteins in transgenic domestic animals may allow an improvement in growth characteristics after a single generation rather than the multiple generations required by standard livestock breeding practices. Additionally, these improved growth characteristics may not require the use of feed additives or other medications. Experience introducing foreign genes into domestic animals has been limited, however. The only published report concerning the introduction of foreign genes into any domestic species used the mouse metallothionein promoter linked to the human somatotropin gene (4). Transgenic pigs carrying this fusion gene had elevated levels of circulating human somatotropin. However, unlike the transgenic mice that expressed the same gene (2), the pigs did not show a significant increase in growth. It is possible that the human somatotropin produced by the transgenic pigs was biologically inactive. Alternatively, the mouse metallothionein promoter may not be optimal for expression of foreign genes in transgenic livestock. Therefore, we sought to determine whether a viral promoter-enhancer would be functional and whether a rat somatotropin gene product would be biologically activ in transgenic pigs. The Moloney murine leukemia virus (MLV) promoter was

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selected because of its efficiency in gene transfer x-periments in a wide variety of cultured cell lines. Additionally, the MLV promoter was predicted to be constitutively active *in vivo*, obviating th need to administer exogenous chemical inducers to the genetically altered animals. Using an MLV-rat somatotropin fusion gene, we produced one transgenic pig which expressed high levels of rat somatotropin. This animal was monitored for phenotypic effects including growth rate, fat production, skeletal growth, and circulating levels of insulin-like growth factor-I (IGF-I). RNA was purified from tissues and used in Northern blot assays to determine the range of expression of the MLV promoter.

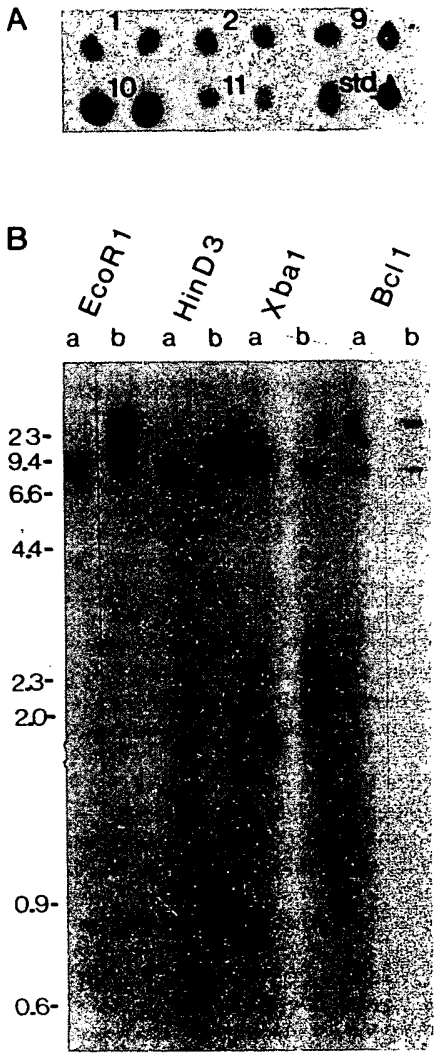

Fig. 1. Identification of Transgenic Swine by Dot Blot Hybridization and Southern Blot Analysis of Genomic DNA

A, Dot-blot from litter 308. Pig 10 contains eight copies per genome of the MLV-rat somatotropin fusion gene. Pigs 1, 2, 9, and 11 show only background hybridization to endogenous somatotropin genes; Std, two copy standard. B, Southern blot. Lane a, Pig 308-11; lane b, pig 308-10; position of *Hin*dIII-digested λDNA and Dde I-digested pUC 12 markers in kb pairs are indicated in the margin.

RESULTS

A total of 100 one-cell and 70 two-cell porcine embryos was collected from 12 Yorkshire gilts. Approximately 2 pl of DNA solution (1.75 ng/μl) containing the Moloney MLV-rat GH (rGH) fusion gene were injected into the male pronucleus of one-cell embryos or into the nucleus of a single blastomere of two-cell embryos.

Of the 15 piglets born, one (no. 308-10) from a litter of four was positive for the MLV-rat somatotropin fusion gene. Dot blot analysis indicated that this pig contained approximately eight copies of the foreign gene (Fig. 1A). Southern blot analysis using DNA extracted from leukocytes of the transgenic pig showed that the endogenous porcine somatotropin gene is represented by an approximately 9-kilobase (kb) fragment in DNA digested with the restriction enzymes *Eco*RI, *Hin*dIII, *Xba*I, and *Bcl*I (Fig. 1B). Electrophoresis of the DNA fragments for a longer period of time indicated that the high molecular weight fragments obtained after restriction endonuclease cleavage were unique for each enzyme. Because the microinjected DNA fragment contains no internal *Eco*RI or *Bcl*I restriction sites, the additional high molecular weight band in the *Eco*RI and *Bcl*I digests indicated a single integration site into the genome of the transgenic pig. *Hin*dIII digestion produced multiple copies of the intact 850 base pair (bp) rat somatotropin cDNA. Digestion with the restriction enzyme *Xba*I resulted in an intensely hybridizing 1900-bp band and a lighter band of 2450 bp which probably represents a junctional fragment. These findings suggest that multiple copies of the MLV-rat somatotropin gene had integrated in a tandem array at a single site within the genome.

Rat somatotropin was measured in serum samples by RIA to determine whether the integrated MLV-rat somatotropin fusion gene was transcribed and translated. The concentration of immunoreactive rat somatotropin in the transgenic pig was 510, 1300, and 500 ng/ml at 2.5, 3.5, and 5.5 months of age, respectively (Table 1). These values were approximately 50-fold higher than in control animals of comparable age. When the pig was killed at 9 months of age its rat somatotropin level was 660 ng/ml.

Despite the high circulating levels of rat somatotropin in the transgenic pig, we could only detect small quantities of the hormone in tissues by RIA. Consequently, we extracted mRNA from many of the tissues and performed Northern blot analyses. These studies showed the highest concentration of rGH mRNA in

Table 1. Rat somatotropin (rGH) and IGF-I levels in plasma from the transgenic pig (308-10) and 31 control pigs (including samples from three negative littermates) averaged over 2.5 to 9.0 months of age

	rGH (ng/ml)	IGF-1 (ng/ml)
Control	15.7 ± 3.39	55.8 ± 7.86
Transgenic	742.5 ± 164.03	379.5 ± 24.78

Levels are the mean ± SE.

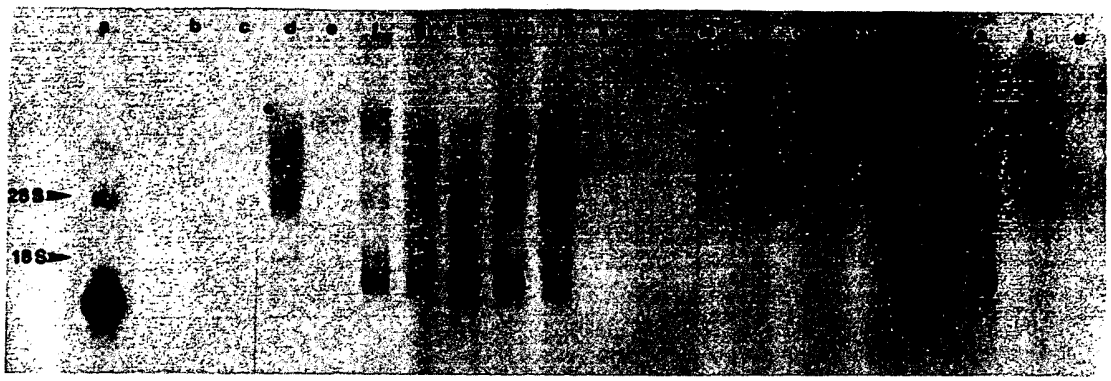

Fig. 2. Northern Blot Analysis of RNA from Pig 308-10

Ten-microgram aliquots of total RNA from each tissue were electrophoresed on a denaturing agarose gel, transferred to a nylon membrane, and hybridized with a radiolabeled rGH cDNA probe. The positions of the 28 S and 18 S ribosomal band are indicated. The predominant hybridizing band in anterior pituitary (lane a) has an estimated size of 1200 bases, consistent in size with the expected polyadenylated porcine GH mRNA. Several other tissues from the transgenic pig have a major hybridizing species of 1600 bases representing the rGH transcript from the MLV promoter. Aside from pituitary, no GH signal was seen in any tissue from control pigs (Low, M. J., unpublished). The lanes are: a, anterior pituitary; b, seminal vesicle; c, testis; d, liver; e, thymus; f, lymph node; g, bone marrow; h, spleen; i, lung; j, kidney; k, adrenal; l, pancreas; m, aorta; n, cerebral cortex; o, tendon; p, tongue; q, stomach; r, colon; s, jejunum; t, skin; u, skeletal muscle.

spleen, lung, colon, and jejunum (Fig. 2). Lymph node, bone marrow, and kidney also contained the fusion gene transcripts, but to a lesser extent. The hybridizing band in the pituitary sample represents the endogenous 1200 nucleotide porcine GH mRNA. Although we were not able to directly compare the levels of pituitary GH mRNA in this transgenic pig to that in controls, subsequent studies have indicated that endogenous GH mRNA is decreased approximately 10-fold in transgenic swine expressing an MLV-porcine somatotropin fusion gene (Ebert, K. M., unpublished). In addition, there was a 10-fold decrease in the number of pituitary cells containing immunoreactive somatotropin in the transgenic swine expressing an MLV-porcine somatotropin gene indicating a negative feedback effect of the ectopic somatotropin on endogenous somatotropin biosynthesis. A similar decrease in endogenous pituitary somatotropin gene expression in transgenic mice was reported previously (5). The predominant band in other tissues has a mol wt of 1600 nucleotides due to the 400 bases of transcribed MLV sequences between the LTR and rat somatotropin cDNA.

IGF-I, a somatotropin-dependent circulating growth factor, was measured in the transgenic pig as an indicator of the biological activity of the rat somatotropin. Serum levels of IGF-I in pig no. 308-10 were 323, 373, 459, and 363 ng/ml at 2.5, 3.5, 5.5, and 9 months, respectively. These values were approximately 7 times that of age-matched controls (Table 1).

Weights were determined every 2 weeks on pig no. 308–10 and the three negative littermates. Despite the high levels of circulating IGF-I, the weight of pig no. 308-10 was only slightly higher than that of the three normal littermates during the rapid growth phase. Th average weights of the three normal littermates at 2.5, 3.5, and 5.5 months of age were 27.9, 45.3, and 94.2 kg, respectively. Th corresponding weights of pig no. 308-10 were 31.6, 48.2, and 104.5 kg, respectively. However, by 9 months of age no. 308-10 was 26% heavier (200 kg *vs.* 159 kg) than the normal male littermate.

Clinical laboratory analysis revealed glycosuria (1000 mg/dl) and a serum glucose level (357 mg/dl) that was approximately 3 times the normal serum level (6). This elevated glucose level was consistent throughout the life span of the animal. Serum electrolytes and liver function studies were normal (data not shown). Testosterone values in the transgenic pig were 6.90 and 2.29 ng/ml at 6.5 and 9.0 months of age, respectively, and were considered normal.

Although the weight of pig no. 308-10 during the rapid growth phase was only slightly increased, several phenotypic changes were evident. Radiographic analyses of the fore and hindlimbs showed increased linear bone growth, increased transverse diameter, and thinned cortices in the metacarpal bones compared to the three control littermates (Fig. 3). Bone age, assessed by epiphysial closure, was not altered. Additionally, the depth of backfat at age 5 months, determined by ultrasonography, was dramatically reduced in the transgenic animal, as compared to the normal male littermate (data not shown). Postmortem examination confirmed the absence of fat (Fig. 4) and revealed extensive joint pathology characteristic of osteochondritis dissecans (7; Fig. 5). Examination of the testes showed tubular atrophy and a marked reduction of spermatogenesis.

DISCUSSION

A transgenic pig containing an MLV-rat somatotropin fusion gene was obtained by microinjecting the gen

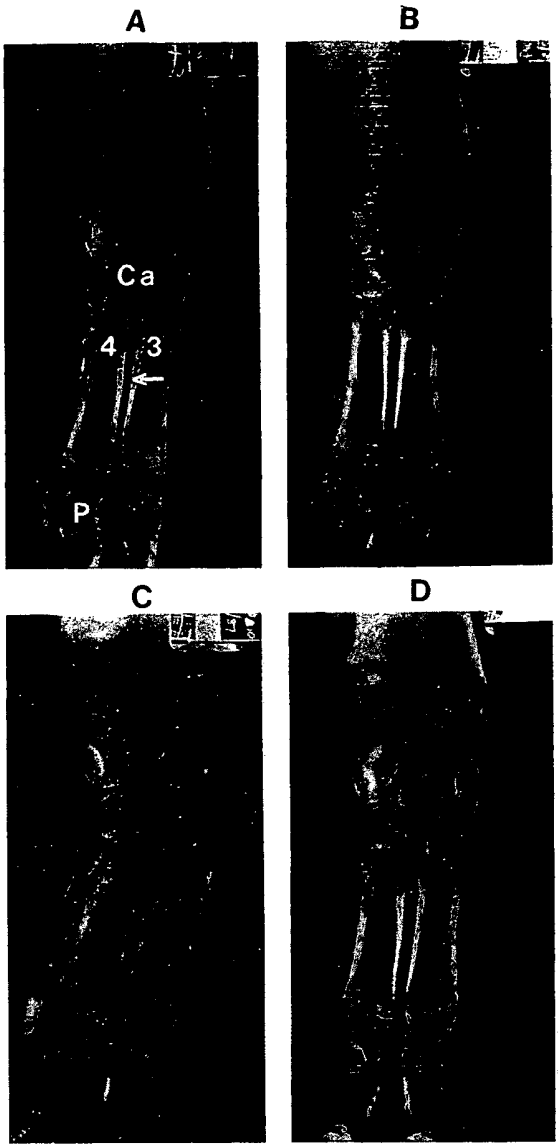

Fig. 3. Radiographic Analyses of the Right Forelimb (Frontal View) of Litter 308

A, 308-1; B, 308-2; C, 308-10; D, 308-11. The x-ray of the transgenic male (308-10) shows an increase in the length and width of the metacarpal bones (3 and 4) and a decrease in the thickness of the cortex (*arrow*). Carpals (Ca) and phalanges (P) are also noticeably larger than in the littermates. Hindlimb x-rays showed essentially the same changes.

into the male pronucleus of a porcine zygote. The gene appeared to be integrated into a unique site within the genome, consistent with observations in transgenic mice (1). The MLV promoter is transcriptionally active in pigs, as shown by the high levels of circulating rat somatotropin. The human somatotropin gene under the control of the mouse metallothionein promoter, has also been shown to be expressed in transgenic pigs (8). Unlike the metallothionein fusion genes however (9), the MLV-fusion gene xamined in our studies was xpressed primarily in hematopoietic tissues and a

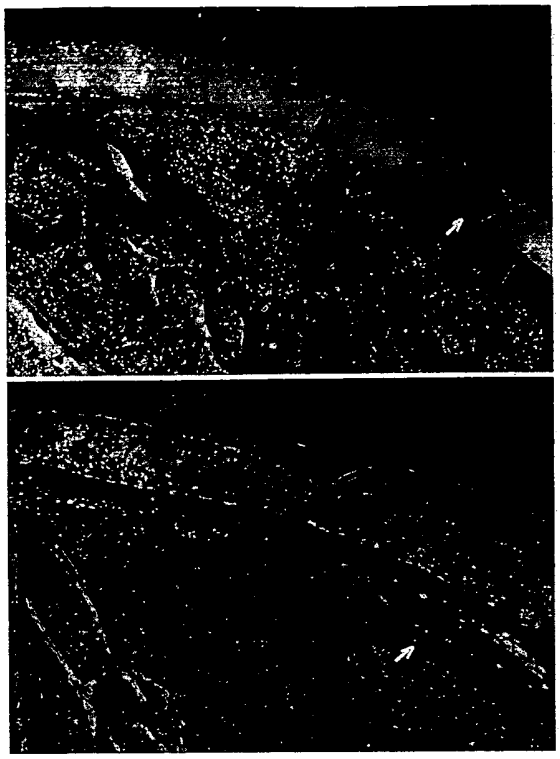

Fig. 4. Cross-Section through the Midlumbar Region of the Transgenic Pig (308-10), Top; and the Nontransgenic Negative Male Littermate (308-11), Bottom

Note the dramatic decrease in the amount of backfat (*arrows*) in the transgenic pig.

subset of nonhematopoietic tissues including kidney, lung, and intestine. This pattern of expression overlaps with the natural tropism of the intact retrovirus in mice. Little expression was detected in liver, the major site of metallothionein-fusion gene expression.

Elevated levels of IGF-I were detected in the transgenic pig indicating that the rat somatotropin was biologically active. This result appears to conflict with an earlier report suggesting that exogenous rat somatotropin given by injection is not biologically active in hypophysectomized swine (10). It is possible that this difference is due to the extremely high and sustained levels of somatotropin produced in the transgenic pig. Previous studies have also shown that injections of human, rat, and even porcine somatotropin only minimally affect weight gain during the rapid growth phase of swine (11, 12). It was not surprising, therefore, that the transgenic pig showed a normal rate of growth during its rapid growth phase (2–6 months). The observation that the transgenic pig continued to gain weight at a continuous rate from 2–9 months of age while the normal littermates decreased their rate of growth after 6 months suggests that continuously elevated levels of rat somatotropin may have the potential to augment the growth of swine during a later phase of development.

The dramatic effects of the rat somatotropin levels on skeletal growth and backfat indicate that major

Fig. 5. The Left Humeral Condyles from the Transgenic Pig 308-10

There were several loose, white cartilage bodies on the glenoid surface (*arrow*), a condition that is indicative of osteochondritis dissecans.

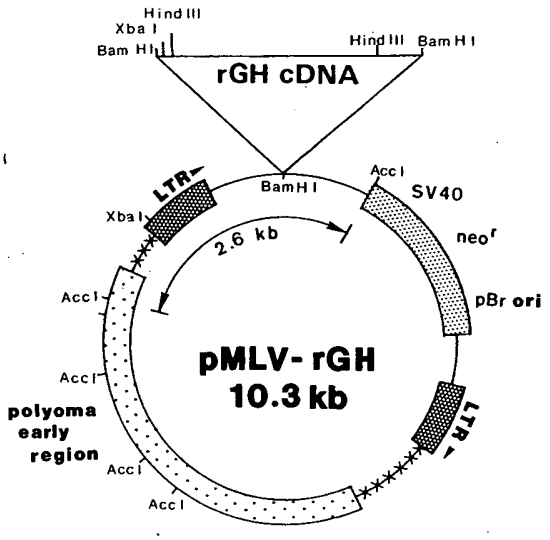

Fig. 6. Structure of the MLV-Rat Somatotropin (MLV-rGH) Fusion Gene

An 850 bp fragment of the rat somatotropin cDNA containing the entire protein coding sequence was ligated into the unique *Bam*HI site of the shuttle vector pDOL downstream of the MLV 5′-LTR. Restriction endonuclease sites used for cloning and Southern blot analysis are indicated. A 2.6-kb *Acc*I fragment indicated by the *double-headed arrow* was used for microinjection into porcine zygotes.

phenotypic changes can occur in transgenic livestock. The reduction in carcass fat within the transgenic pig is similar to the fat depletion seen in pigs injected daily with porcine somatotropin (13) and is probably due to a direct action of somatotropin itself. It is possible, however, that the decrease in fat deposition is related in part to the coincidental diabetes mellitus. The increase in long bone growth and the joint pathology may be due to either the elevated levels of somatotropin or IGF-I. Similar but less dramatic osteochondritis has been detected in several nontransgenic animals from our herd at comparable age and may indicate a normal condition which is enhanced by elevated GH (7). Also, identical phenotypic changes (*i.e.* decreased fat deposition and joint pathology) have been noted in other transgenic pigs expressing an MLV-porcine somatotropin fusion gene (Ebert, K. M., unpublished).

Chemical profiles of the transgenic animal also show typical endocrine effects of high levels of somatotropin. Elevated blood glucose is a common feature of acromegaly in humans and is thought to be due to insulin resistance.

Our studies indicate that major phenotypic changes can be produced in transgenic livestock through expression of microinjected fusion genes. Overproduction of rGH in the transgenic pig led to several desirable, and some clearly undesirable new traits. The full potential of this technology will be realized only when it becomes possible to precisely regulate the expression of the microinjected genes.

MATERIALS AND METHODS

Construction of the MLV-rGH Fusion Gene

An 850 bp *Hin*dIII restriction fragment of the rat somatotropin cDNA containing the entire protein coding sequence (14) was adapted for ligation into the unique *Bam*HI site of the shuttle vector pDOL (15) downstream of the MLV 5′-LTR (Fig 6).

*Bam*HI cohesive ends were obtained by initially cloning a 1470 bp *Cla*I-*Acc*I fragment containing the rat somatotropin cDNA into the plasmid pUC 12, and then isolating the rat somatotropin sequence by *Bam*HI digestion. In the resulting 1200-bp *Bam*HI fragment, the rat somatotropin cDNA is flanked by a short polylinker sequence at its 5′-end and by 345 bp of pBR322 at the 3′-end. Restriction endonuclease sites used for cloning and Southern blot analysis are indicated in Fig. 6. A 2.6-kb *Acc*I fragment was used for microinjection into the porcine zygotes.

Production of Transgenic Pigs

Twelve Yorkshire gilts were used for collection of one-cell and two-cell porcine embryos. The gilts were synchronized and superovulated with a modified feeding regime of Webel (16). The animals were fed a synthetic orally active progestin, Altrenogest (Regu-Mate, Hoechst, West Germany), at a concentration of 15 mg in 1.8 kg feed/day for 9 days beginning on days 12–16 of the estrous cycle. Ovulation occurred approximately 5 days after the last feeding of progestin. Gilts were superovulated by injecting sc 2000 U PMSG (Sigma, St. Louis, MO) 24 h after the last feeding of Altrenogest, followed 78 h later with an im injection of 1000 U human chorionic gonadotropin (hCG, Sigma). This regime produced an average of 28 ovulations per animal. Gilts were bred by artificial insemination with sperm from proven boars at 24, 36, and 48 h after injection of hCG.

Embryos were collected from gilts under aseptic conditions by cannulating the ampulla region of the oviduct and flushing the oviduct with 10–15 ml Kreb's Ringer-Bicarbonate solution. The embryos were initially maintained at 30 C, then were transferred to Ham's F12 medium containing 10% fetal calf serum and were incubated at 38 C in 5% CO_2. Before injection, the zygotes or two-cell embryos were centrifuged at 13,000 × *g* in an Eppendorf centrifuge to visualize the pronuclei or nuclei, respectively. The male pronucleus or one nucleus of the two-cell embryo was injected with approximately 2 pl plasmid solution using interference contrast microscopy. The injection was done in a droplet of modified BMOC-2 medium containing HEPES salts (17). The injected zygotes were stored in an incubator at 38 C until transfer to a recipient female.

DNA Ear Blot Hybridization

DNA was extracted from an ear punch (18), quantified using the Hoechst dye method (19), and 1.8 μg were dotted in duplicate onto nitrocellulose. The dot blot was probed with a cDNA encoding the porcine somatotropin coding sequences (Mandel, G., unpublished), radiolabeled to a specific activity of 10^9 cpm/μg by random hexanucleotide priming (20). This cDNA insert, confirmed by nucleotide sequencing (21), hybridizes equally well to rat and porcine somatotropin genomic sequences. Two copies of the integrated gene were calculated to be equivalent to 0.5 pg of an 850-bp rat somatotropin cDNA restriction fragment. Copy number in the pigs was determined by scintillation counting of the DNA dot pairs and comparison of counts obtained from genomic DNA to the standards. A normal pig was assumed to contain two alleles of a single copy somatotropin gene.

Southern Blot Analysis of the MLV-rGH Fusion Gene

High molecular weight DNA was extracted from peripheral leukocytes (22) and 10-μg samples were digested to completion with the indicated restriction endonucleases. The samples were electrophoresed on a 0.7% agarose gel for 15 h at 50 V, transferred by electroblotting to a Zetabind membrane, and prehybridized in 6× SSC, 1% sodium dodecyl sulfate, (SDS), and 50 μg heparin/ml at 65 C for 1 h.

Hybridization was performed in 6× SSC, 1% SDS, and 500 μg heparin/ml using 1.5×10^6 cpm porcine somatotropin cDNA probe/ml. The filter was washed in 0.2× SSC, 0.2% SDS at 65 C, and autoradiographed for 24 h with an intensifying screen.

Northern Blot Analysis of the rGH mRNA

Total cellular RNA was prepared from frozen tissues by extraction in 5 M guanidine thiocyanate, 20% β-mercaptoethanol (vol/vol), 10 mM EDTA, 50 mM Tris-HCl, pH 7,5, followed by precipitation in 4 M LiCl (23). The quality of the RNA was checked by ethidium bromide staining of the 28 S and 18 S ribosomal bands on a native agarose gel. Ten micrograms of total RNA were electrophoresed on a 1% agarose-formaldehyde gel and transferred by electroblotting to a Zetabind (American Bioanalytical, Natick, MA) membrane. Prehybridization and hybridization conditions were performed as previously described (24). A rat somatotropin cDNA probe was radiolabeled as described above. The molecular weight of hybridizing bands was estimated from the positions of the 28 S and 18 S ribosomal bands and an RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD).

RIAs

Circulating rat somatotropin was measured by RIA according to Glenn (25). The cross-reactivity of the antibody with porcine somatotropin was 60%. Serum samples were taken at 1-month intervals beginning at 2 months of age until the animal was killed. As only one blood sample was taken at each time, the fluctuations in endogenous somatotropin were not considered and blood samples from 31 control pigs including samples from three negative littermates were averaged over 2.5 to 5.5 months of age for a control value.

IGF-I was measured in the same samples using a heterologous human IGF-I RIA as outlined by Buonomo *et al* (26).

Acknowledgments

The authors would like to acknowledge A. Schmidt, T. Smith, S. Hagopian, R. Felix and C. Banks for their excellent technical assistance. We would also like to thank Dr. J. Erickson, G. Miller and K. Baldwin for their help with the synchronization, superovulation, and artifical insemination of the swine and animal husbandry that is so critical to a successful program with research using farm animals, and Drs. G. Meuller and M. S. A. Kumar for the Northern Blot and testosterone measurements, respectively. The authors would also like to acknowledge the Veterinary staff at the Tufts University School of Veterinary Medicine, especially Drs. H. Ernst and S. Schelling, for their assistance in the medical aspects of this study. Acknowledgment is also extended to J. Ebert for assistance with the preparation of this manuscript. The authors have complied with the appropriate experimental procedures for the care and use of animals as stated by the *American Journal of Physiology*.

Received November 9, 1987. Accepted December 16, 1987.

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This work was funded by a grant from the Monsanto Company.

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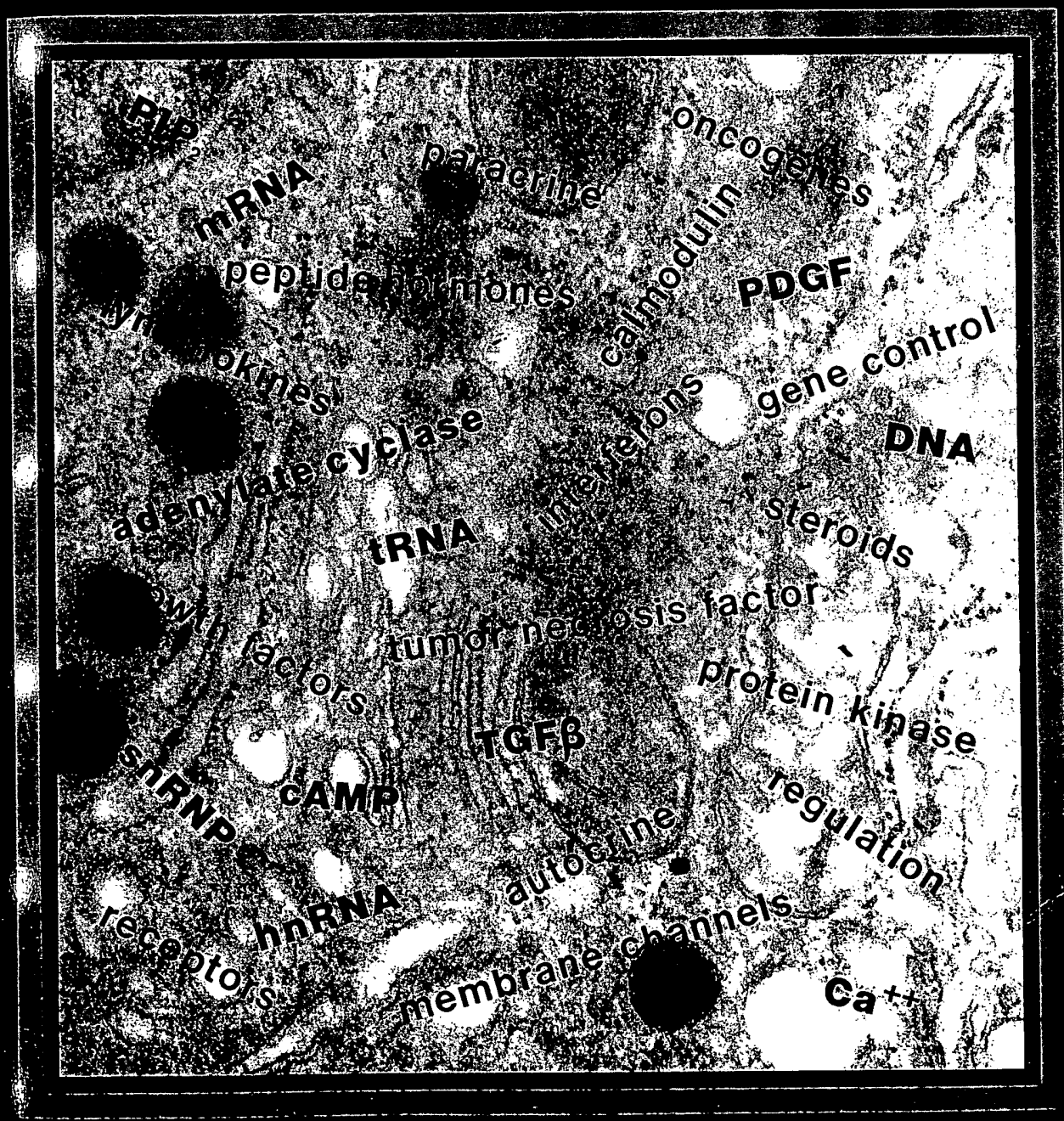
MOLECULAR ENDOCRINOLOGY

Volume 2, Number 3 March 1988



ISSUED MONTHLY FOR THE ENDOCRINE SOCIETY

Formation f β-amyloid protein deposits in brains of transgenic mice

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DEPOSITS of β-amyloid are one of the main pathological characteristics of Alzheimer's disease. The β-amyloid peptide constituent (relative molecular mass 4,200) of the deposits is derived from the β-amyloid precursor protein (β-APP) which is expressed in several different isoforms[1-6]. The two most prevalent β-APP isoforms are distinguished by either the presence (β-APP751) or absence (β-APP695) of a Kunitz serine protease inhibitor domain. Changes in the abundance of different β-APP messenger RNAs in brains of Alzheimer's disease victims have been widely reported[7-12]. Although these results have been controversial, most evidence favours an increase in the mRNAs encoding protease inhibitor-containing isoforms of β-APP and it is proposed that this change contributes to β-amyloid formation[9-12]. We have now produced an imbalance in the normal neuronal ratio of β-APP isoforms by preparing transgenic mice expressing additional β-APP751 under the control of a neural-specific promoter. The cortical and hippocampal brain regions of the transgenic mice display extracellular β-amyloid immunoreactive deposits varying in size (<5–50 μm) and abundance. These results suggest that one mechanism of β-amyloid formation may involve a disruption of the normal ratio of neuronal β-APP isoform expression and support a direct relationship between increased expression of Kunitz inhibitor-bearing β-APP isoforms and β-amyloid deposition.

A chimaeric gene was constructed between the human β-APP751 complementary DNA and the rat neural-specific enolase (NSE) promoter, termed NSE: β-APP751. The rat NSE promoter directs the neural-specific expression of β-galactosidase in transgenic mice[13] and we have confirmed this using a NSE promoter fragment slightly truncated at the 5′ terminus (our unpublished results). The promoter fragment containing the 5′ untranslated region of NSE and a roughly 1.2-kilobase (kb) intron in this domain was fused to the β-APP751 cDNA such that the initiator methionine of NSE was replaced with the initiator methionine of β-APP751. Nine of 44 mice that developed from embryos injected with NSE:β-APP751 DNA carried the transgene. Three pedigrees were selected for extensive characterization: founders 10 (F10), 11 (F11) and 24 (F24). Homo- and hemizygotic states and transgene copy numbers were determined by comparison to the endogenous single copy β-APP mouse gene using Southern blot hybridization with a probe common to both mouse β-APP and human β-APP751 (Table 1).

RNA expression of the inherited transgenes in the three pedigrees was investigated. Total brain RNA was isolated both from positive and from wild-type control animals, reverse transcribed, and a specific DNA subfragment amplified by polymerase chain reaction (PCR). Primers for PCR were designed such that only transcripts derived from the transgene would be amplified, that is, one primer hybridizes to the NSE 5′ untranslated region and the other to the 5′ coding domain of β-APP. The NSE PCR primer corresponds to a site located upstream of the intron so that amplification of contaminating genomic DNA or unprocessed transcripts could be detected. A predicted 373-base pair (bp) fragment is amplified from reverse-transcribed RNA prepared from each transgenic animal but not from wild-type mice (Fig. 1*a*). As a control, half of the reverse-transcribed RNA was amplified with a primer for the native

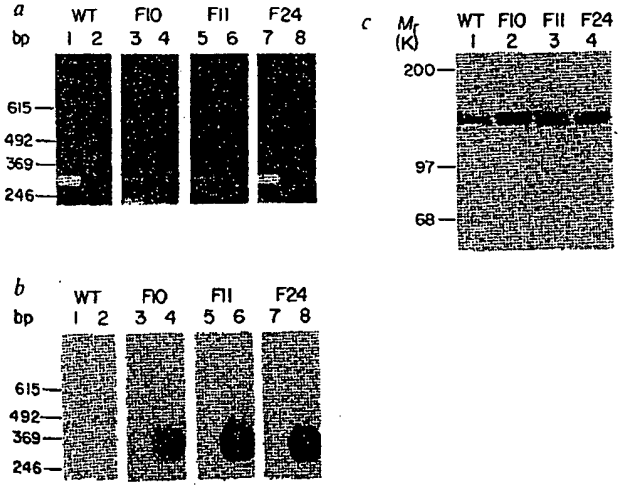

FIG. 1 NSE: β-APP751 expression in brain. *a*, Agarose gel electrophoresis of reverse transcribed PCR products visualized with ethidium bromide staining. Even numbered lanes, reverse transcribed PCR products from wild-type (WT), NSE: β-APP751 founder 10 (F10), founder 11 (F11) and founder 24 (F24) RNA primed with primers specific for NSE: β-APP751 RNA. Odd numbered lanes, reverse-transcribed PCR products from RNA primed with primers specific for endogenous β-APP RNA. *b*, Southern blot of above reverse-transcribed PCR products using ^{32}P-labelled oligonucleotide probe specific for the NSE: β-APP751 chimaeric gene. Lanes are the same as in *a*. *c*, Western blot analysis of β-APP in brain. Lane 1, WT; 2, F10; 3, F11; 4, F24 total brain protein immunoblotted with β-APP antiserum.

METHODS. An ~8-kb rat genomic fragment containing the NSE gene was isolated on the basis of the published sequence[22] and the 2.3-kb fragment used for the chimaeric gene isolated by PCR. PCR primers were designed to generate *Bgl*II and *Nco*I sites at the 5′ and 3′ terminus of the fragment, respectively. A *Nru*I(position 123)–*Xmn*I(position 2,665) fragment was isolated from β-APP751 cDNA[4], and was ligated with the 2.3-kb NSE fragment harboured in a derivative of pcDV1 plasmid containing the simian virus 40 (SV40) late region polyadenylation signal[23]. A linear fragment of NSE: β-APP751 was prepared by cleavage with *Sal*I and *Nde*I and was injected into fertilized embryos of the JU strain of mouse[24]. The JU strain, developed by Eric Bradford at the University of California Davis, was chosen for its large litter size. For genotype and copy number determinations, 40 μg of tail DNA was digested with *Bgl*II and electrophoresed on 0.8% agarose gels. Southern blots were prepared[25] and hybridized with an oligonucleotide probe (5′-ATGGATGTGTACTGTTTCTTCTTCA-3′) radiolabelled with ^{32}P by T4 kinase. Blots were hybridized at 60 °C in 6×SET (1×SET=0.15 M NaCl, 30 mM Tris-HCl pH 8.0, 2 mM EDTA) with 5×Denhardt's solution and washed at 60 °C for 40 min in 6×SSC (1×SSC=0.15 M NaCl, 0.015 M Na-citrate). For transcriptional analyses, 2 μg of total brain RNA was reverse transcribed with oligo(dT)$_{12-18}$, after which the reaction was divided into two equal aliquots. PCR[26] was done on one aliquot using NSE (5′-CACCGCCACCGGCTG-AGTCTGCAGTCCTCG-3′) and β-APP (5′-TCTTGCACTGCTTGCGGCCCCGCTT-GCACC-3′) primers and on the second aliquot with the same β-APP primer and a primer to the secretory signal sequence of β-APP (5′-TTGGCACTGCT-CCTGCTGGCCGCCTGGACG-3′) in place of the NSE primer. DNA products were electrophoresed on 2% agarose gels, visualized by staining with ethidium bromide, then Southern blotted[25] and hybridized with a ^{32}P-labelled oligonucleotide probe to the NSE: β-APP751 fusion sequence (5′-AGATCCCAGC-CACCGATGCTGCCCGGTTTG-3′). Blots were hybridized at 65 °C in 6×SET and washed at 65 °C for 40 min in 4×SSC. Protein homogenates were made from total brain[14] and 50 μg of each sample was electrophoresed on 7.5% SDS–polyacrylamide gels[27]. A western blot[28] was developed using a 1:500 dilution of antiserum and ^{125}I-labelled protein A. The antiserum was raised against full-length human β-APP695 expressed by a recombinant vaccinia virus[16]. Identically prepared gels stained with Coomassie blue dye confirmed that equivalent amounts of protein were loaded for each sample.

β-APP secretory signal sequence and the same 5′ coding domain β-APP primer to produce a 307-bp DNA fragment representing amplification from endogenous β-APP RNA (Fig. 1*a*). When all the PCR reaction products are hybridized with a probe bridging the junction between NSE and β-APP751 sequences, only the products derived from the transgenic brains hybridize, documenting the authenticity of the 373-bp PCR product (Fig. 1*b*).

Western blots were made to evaluate changes in protein expression in the brains of the transgenic animals. Equal amounts of total protein from whole brain homogenates were electrophoresed on polyacrylamide gels, transferred to a membrane then reacted with polyclonal serum raised against full-length β-APP. A band (or set of unresolved bands) of relative molecular mass of about 130,000 (130K), corresponding to the reported average size of mammalian brain β-APP isoforms[14–16], is observed in the control, as well as in each transgenic protein homogenate (Fig. 1*c*). This signal is increased in the NSE:β-APP751 samples relative to the wild-type sample suggesting globally elevated β-APP751 expression in the transgenic brains. To obtain a more refined examination of NSE:β-APP751 expression and its effects, we used immunocytochemistry.

A panel of monoclonal antibodies was prepared using a synthetic peptide corresponding to residues 1–28 of the β-amyloid protein as the immunogen. The specificity of the monoclonals was established by immunoperoxidase staining of brain sections from Alzheimer's disease victims (Fig. 2 *a*, *b*). Sections were prepared from brains of NSE:β-APP751 transgenic mice, as well as from wild-type mice and both were stained in parallel with one monoclonal, 4.1 (Table 1). Reproducibly greater immunoperoxidase reactivity is observed in neurons and as fine puncta throughout the neuropil of the transgenic brains compared with the immunoreactivity visualized in brains from wild-type mice. A pronounced staining of neuritic processes is also noticeable (Fig. 2*d*). This enhancement of arbor-forming neuronal processes is most evident in the stratum flanking the pyramidal cell layer of the CA-1 and CA-3 regions of transgenic hippocampi. Both neuronal and process staining are fully competed by earlier incubation of the antibody with the synthetic β-amyloid peptide. Neuritic staining in the transgenic brains is also detected using antibodies raised against full-length β-APP (Fig. 2*c*), indicating full-length β-APP is present in the neuritic

TABLE 1 Summary of mice used for immunohistology

Line	Animal	Sex	Age*	Genotype	Copy number†	Deposits‡
NSE:β-APP751						
10	0	F	12	Aa	1	+++
	31§	F	7	Aa		++
	168	F	5	Aa		+++
	334	M	2	AA		+
11	0	M	15	Aa	4	+++
	51	M	12	Aa		+
	236	M	4	AA		+++
	287	F	3	AA		+
24	77	M	8	Aa	8	+
	201	F	5	AA		+
Wild type						
	1	F	4	NA	NA	—
	2	F	4			—
	3	M	5			—
	4	M	3			—
	5	M	9			—
	6	F	12			—
	7	M	14			—

M and F indicate male and female mice, respectively; AA and Aa represent homozygotic and hemizygotic animals, respectively; NA, not applicable.
* Months.
† Haploid.
‡ >5 μm in size, (−) none, (+/++/+++) relative abundance of deposits, that is, + indicates <5; ++ indicates 5-10; +++ indicates >10 deposits per section as an average of multiple sections stained.
§ NSE: β-APP751 F10, number 31 died of unknown cause.

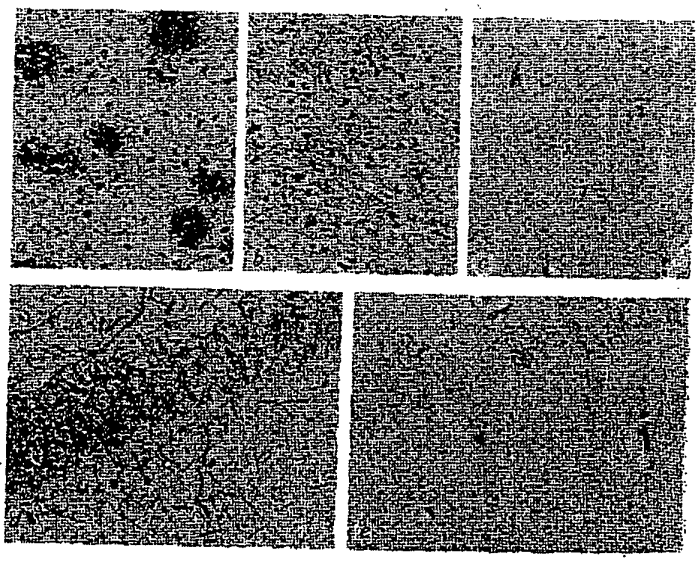

FIG. 2 Immunoperoxidase staining of human and mouse brain. Human Alzheimer's disease tissue section from caudal hippocampus stained with 4.1 antibody without (*a*) and an adjacent section (*b*) with preincubation with the β-amyloid synthetic peptide immunogen (×250). Hippocampal CA-1 field of NSE: β-APP751 F10 (number 334) stained with antibodies to full-length β-APP (*c*); pyramidal cell layer of CA-1 region from NSE: β-APP751 F10 (number 334) stained with 4.1 antibody (*d*); same region from wild-type mouse (number 3) identically stained with 4.1 antibody (*e*) (×500).

METHODS. A synthetic peptide corresponding to residues 1–28 of the β-amyloid protein[1,2] was prepared and self-aggregated by freezing and thawing. The peptide aggregate was mixed with methylated bovine serum albumin and adjuvant for immunizing and boosting mice. Hybridomas from the sensitized spleen cells were generated[29]. Clones secreting anti-peptide antibodies were expanded and subcloned by limiting dilution. The epitope recognized by each monoclonal was mapped to the N-terminal 10 residues of the β-amyloid protein. For analysis of mouse brains, brains were removed and fixed with 4% paraformaldehyde, embedded in paraffin and 6 μm coronal midbrain sections made. Sections were deparaffinized, rehydrated, treated for 30 min with 0.3% H_2O_2, then with 80% formic acid for ~2 min. Sections were next incubated at 37°C for 30 min with a 1/20 dilution of conditioned medium from the hybridoma secreting the 4.1 antibody. An anti-mouse avidin-biotinylated horseradish peroxidase (ABC) kit was used according to supplier's recommendations (Vector, Burlingame, California) and the horseradish peroxidase visualized with 3,3′-diaminobenzidine. Staining for full-length β-APP used the antiserum used for western blotting in *c* at a 1:500 dilution and an anti-rabbit ABC kit. Formic acid treatment was omitted for β-APP staining. Sections were counterstained with haematoxylin and eosin. Human brain sections were from individuals clinically diagnosed with Alzheimer's disease. The human sections were prepared and stained identically as mouse tissue sections except they were treated with 98% formic acid for 10 min. For competition experiments, the antibody diluent was preincubated at 4 °C for 12 h then 37 °C for 30 min with 250 μg ml^{-1} 1–28 β-amyloid synthetic peptide before application. Congo red, thioflavin S and silver staining were done using published procedures[18–21,30].

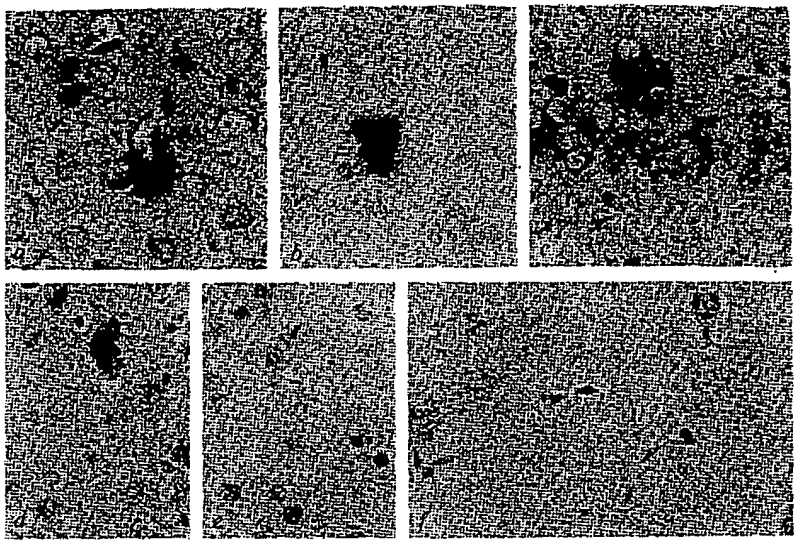

FIG. 3 Immunoreactive deposits in NSE: β-APP751 brains. *a*, Compact deposit in frontalparietal cortex of F11 (number 0); *b*, compact deposit in thalamus of F11 (number 236); *c*, compact deposit in hippocampal CA-2 field of F11 (number 0); *d*, cluster of deposits in frontalparietal cortex of F10 (number 168); *e*, adjacent section as in *d* but antibody preincubated with β-amyloid synthetic peptide before staining (arrowheads demark same capillaries in field of *d* and *e*); *f*, amorphous deposits in the hippocampal stratum moleculare of F11 (number 236) (×500). Immunocytochemistry and competition were performed as described in the legend to Fig. 2.

processes, although the possibility that the neurites also contain β-amyloid protein cannot be excluded.

Extracellular immunoreactive deposits are also consistently seen in the brain sections from each of the three transgenic lines stained with the 4.1 monoclonal which are not seen in sections from wild-type animals identically stained. The immunoreactive deposits vary in size, shape and frequency. Examples of compact deposits 10–50 μm in diameter are shown in Fig. 3*a–d*. These immunoreactive deposits tend to occur in clusters and are most frequently observed in the cortex and hippocampus although occasionally they have been found in the thalamus and striatum. A second type of immunoreactive extracellular deposit is reproducibly seen in transgenic brain sections which is lacking in control brain sections. This type of deposit is diffuse, amorphous and granular (Fig. 3*f*). Detection of extracellular deposits in the tissue sections from the transgenic animals required treatment with formic acid. Immunoreactivity of these structures also could be competed by the β-amyloid peptide (Fig. 3*e*). In a preliminary survey, antibodies to full-length β-APP stained extracellular deposits of similar morphology as those stained by the 4.1 monoclonal. Also, in general, the deposits are stained by silver salts, infrequently by thioflavin S, but not by Congo red, corroborating a preamyloid-like composition (data not shown). Owing to the small group of animals analysed, it is difficult to make a correlation between the frequency of deposit appearance with age, genotype or sex (Table 1).

Although there may exist several different mechanisms promoting β-amyloid formation[17], the observed increased level of Kunitz inhibitor-containing β-APP isoform RNA in neurons of Alzheimer's disease brains suggests that Kunitz inhibitor β-APP isoform overexpression may be one mechanism[9–12]. The three NSE:β-APP751 transgenic lines which have moderately increased neuronal expression of β-APP751 and form extracellular β-amyloid immunoreactive deposits (as well as our preliminary data on NSE:β-APP695 transgenic lines which do not) support this hypothesis. The two types of extracellular deposits, diffuse and compact, seen in the transgenic mice resemble several β-amyloid structures typically seen in the brains of Alzheimer's disease victims, specifically preamyloid and preamyloid plaques[18–21]. It will be of interest to determine whether the quality and/or quantity of deposits change in an age-dependent manner and if the mice display other pathological features characteristic of Alzheimer's disease. □

Received 31 January; accepted 25 June 1991.

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ACKNOWLEDGEMENTS. We thank G. Anderson, S. Donohue and A. Moyer for performing transgenic embryology, A. Lam, U. Masharani, L. Carstensen and P. Hummel for their technical assistance and contributions in the development and care of transgenic animals, T. Finch for providing human brain sections, G. Murphy for viewing stained tissue sections, and E. Stoelting for artwork. This work was supported by Daiichi Pharmaceutical Co. of Tokyo, Japan.

Spontaneous calcium release from inositol trisphosphate-sensitive calcium stores

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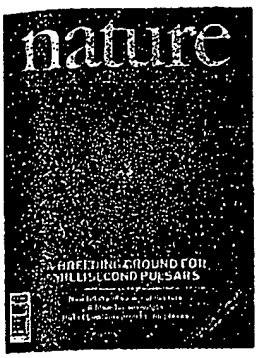
Inositol 1,4,5-trisphosphate ($InsP_3$) functions as a second messenger to mobilize Ca^{2+} from intracellular reservoirs[1]. The release mechanism displays all-or-none characteristics[2,3], that may account for other bservations that the $InsP_3$-induced mobilization of Ca^{2+} is quantal[4–6]. Quantal release may depend on the sensitivity of the $InsP_3$ receptor being regulated by the Ca^{2+} concentration in the lumen of the endoplasmic reticulum[7]. We report here that



nature

11 July 1991

Vol. 352 Issue no. 6332

◀ The second brightest globular cluster in the sky, NGC104 or 47 Tucanae, is now shown to host not one but 11 millisecond pulsars (pages 219 and 195), bringing the present count to 23. 'Wind' from such pulsars could explain the relative lack of gas in globular clusters, page 221. (Photo: ESO.)

THIS WEEK . . . THIS WEEK . . . THIS WEEK . . .

thirtysomething

Alkali-metal-doped compounds of C_{60} fullerenes are proving fertile ground for the search for high-temperature superconductors. Potassium and rubidium doping are known to produce superconducting phases, but now two labs report superconductivity at and above 30 K using caesium (page 223) and caesium/rubidium dopants (see page 222).

Seventh gear

Development of the R7 photoreceptor in the *Drosophila* eye involves an interaction requiring expression of the cell surface receptor encoded by *sevenless* gene in R7 cells and *bride of sevenless* in R8 cells. On page 207, Krämer *et al.* demonstrate that 'boss', a protein on the surface of R8, interacts directly with sev on R7, and that the boss protein is actually taken up by the R7 cells by receptor-mediator endocytosis. News and Views page 193.

Rb function

The function of the product of the retinoblastoma gene in normal cells is a negative regulator of cellular proliferation. Wild-type retinoblastoma protein forms a complex with a cellular transcription factor; on page 249 this complex is shown also to contain cyclin A and a naturally occurring mutant of *Rb* is reported to be unable to form this complex. In a separate experiment (page 251), the cloning of genes encoding two human cellular proteins that bind to the *Rb* product in competition with DNA tumour virus transforming proteins is reported. News and Views page 189.

Fish course

The earliest-known land vertebrate, *Acanthostega*, had fish-like internal gills, suggesting that it retained far more of an aquatic lifestyle than has been realized. Page 234.

Sulphur count

Radioactive sulphur-35, produced by cosmic-ray bombardment in the upper atmosphere, provides a marker with potential as a worldwide measure of SO_2 oxidation and sulphate removal rates. Aerosol sulphate is thought to play a role in tropospheric heat balance, and is therefore relevant to the issue of global warming. See page 226.

Fatherly figures

Strict maternal inheritance of mitochondrial DNA is the norm in animals, but using interspecific mouse backcrosses and PCR techniques, Gyllensten *et al.* are able to show that sperm contribute approximately 1/1,000 of the total mitochondrial DNA to the zygote. Page 255.

Alzheimer model

Transgenic mice into which the gene encoding a putative pathological isoform of the β-amyloid protein has been introduced develop lesions resembling those seen in human Alzheimer's disease. Page 239.

Natural selection

Synthesis gas, a mixture of hydrogen and carbon monoxide, is the starting material for the industrial preparation of many organic compounds. A new strategy for converting natural gas to synthesis gas offers the prospect of a synthesis route with some engineering advantages. Page 225.

Speech recognition

Cochlear implants can give profoundly deaf subjects some hearing ability by converting sound into direct electrical stimulation of the auditory nerve. A new processing strategy gives a significant improvement in the ability of subjects with cochlear implants to recognize speech. Page 236.

Guide to Authors

Inside back cover.

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tisense 1220) were used for the initial amplification, and K3 5′-TAGAACGATTCGCAGTTAAT (*gag* sense 913) and K7 5′-CCTGGATGTTCTGCACTATA (*gag* antisense 1207) were used for the nested amplification. The transferred product was probed with a fragment of pHXB2D (nucleotides 631 to 1258) labeled with deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanidine triphosphate by the random priming method to a specific activity of 1.14×10^8 cpm per milligram of DNA.

32. Supported by PHS program projects NS-27405, NS-11037, and CA-45690, and a grant from the W. W. Smith Charitable Trust (to F.G.-S.). S.B. is also supported by a grant from the National Multiple Sclerosis Society, and S.L.S. is a Mallinckrodt Scholar. We thank B. Godfrey for raising antibody 8586, D. Kolson, R. Collman, and T. Brown for fluorescence microscopy, L. Lynch for technical assistance, C. Griot for providing MAb 16G1, P. McGonigle for help with the Scatchard plot, and J. Hoxie, S. Miller, N. Nathanson, A. K. Asbury, and members of the Nathanson/Gonzalez laboratories for helpful comments and encouragement. We also thank J. Burns (University of Utah).

12 December 1990; accepted 24 May 1991

Deposits of Amyloid β Protein in the Central Nervous System of Transgenic Mice

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Alzheimer's disease is characterized by widespread deposition of amyloid in the central nervous system. The 4-kilodalton amyloid β protein is derived from a larger amyloid precursor protein and forms amyloid deposits in the brain by an unknown pathological mechanism. Except for aged nonhuman primates, there is no animal model for Alzheimer's disease. Transgenic mice expressing amyloid β protein in the brain could provide such a model. To investigate this possibility, the 4-kilodalton human amyloid β protein was expressed under the control of the promoter of the human amyloid precursor protein in two lines of transgenic mice. Amyloid β protein accumulated in the dendrites of some but not all hippocampal neurons in 1-year-old transgenic mice. Aggregates of the amyloid β protein formed amyloid-like fibrils that are similar in appearance to those in the brains of patients with Alzheimer's disease.

ACCUMULATION OF AMYLOID β PROTEIN is a characteristic and diagnostic feature of brains from individuals with Alzheimer's disease (AD) and Down syndrome (DS) (*1*). The 4-kD amyloid β protein is a truncated form of a larger amyloid precusor protein (APP), which has features typical of a cell surface integral membrane glycoprotein (*2*). At least five different APP isoforms containing 563, 695, 714, 751, and 770 amino acids (*3*) can be generated by alternative splicing of primary transcripts of a single gene on chromosome 21 (*3*). The 40- to 42-amino acid β protein segment comprises half of the transmembrane domain and the first 28 amino acids of the extracellular domain of APP (*2*), and is encoded within two exons (*4*).

The mechanism by which the amyloid β protein is derived from its precursor is not known. APP is processed in vitro by a proteolytic cleavage within the amyloid β protein region (*5*). Generation of the amyloid β protein, therefore, involves an alternative processing pathway, possibly as a result of post-translational modifications such as phosphorylation (*6*).

Although the deposition of amyloid appears to be an early event in the progression of AD (*7*), its role in neurodegenerative processes remains unknown. Amyloid β protein can be neurotrophic for undifferentiated hippocampal neurons in culture and, at high concentrations, neurotoxic to differentiated neurons (*8*). Mutant forms of APP have been implicated in hereditary cerebral hemorrhage with amyloidosis of Dutch origin (*9*) and in at least two families with familial forms of AD (*10*). In addition, overexpression of one or more forms of APP may be responsible for the AD-like pathologies of individuals with DS (*11*). These findings suggest that accumulation of amyloid β protein may be a critical step in the neurodegenerative processes of AD.

The lack of experimental animal models for AD has limited the elucidation of the mechanism of amyloid formation and its role in the pathogenesis of AD. Nonhuman primates provide the only in vivo model for investigating amyloid formation in the central nervous system (CNS) (*12*). The high cost and limited availability of aged primates, however, restricts their use as practical model systems. Transgenic rodent models may provide a useful alternative. The expression of native or mutant forms of APP in transgenic mice may help to identify aberrant APP processing pathways that lead to the accumulation of amyloid β protein and clarify the role of amyloid β protein in neuronal degeneration. We therefore initiated a series of experiments to express various forms of APP in the brain of transgenic mice.

We have introduced into mice a construct that encodes the 42-amino acid amyloid β protein, regulated by a 4.5-kb fragment from the 5′ region of the human APP gene (Fig. 1). This APP regulatory region directs neuron-specific expression of the reporter gene *lac Z* from *Escherichia coli* in the CNS of transgenic mice in a pattern that is similar to the pattern of endogenous mouse and human APP mRNA expression (*13*).

Two lines of transgenic mice, AE101 and AE301, expressed human amyloid β protein mRNA in the brain (Fig. 2) and transmitted the transgene in a Mendelian fashion. Steady-state amounts of the transgene mRNA were lower than steady-state amounts of the endogenous mouse APP mRNA. In both transgenic lines, however, human amyloid β protein was synthesized and accumulated in the CNS of 1-year-old mice (Fig. 3).

We examined immunocytochemical and ultrastructural features of brains from several F1 generation transgenic mice from lines AE101 and AE301 at approximately 1 year of age. When sections of brain from 1-year-old control mice were stained with antibodies to the amyloid β protein (*14*), no immunoreactivity was detected (*15*). In contrast, sections of brain from transgenic mice showed amyloid β protein immunoreactivity (Fig. 3). Amyloid β protein staining was located predominantly in the hippocampus, where it appeared as clusters of dots that were symmetrically distributed on both sides of the brain. Within the hippocampus, amyloid β protein immunoreactivity was most prominent in the molecular layer of CA1 and CA2; only occasional amyloid β protein–positive clusters were detected in CA3 regions of the hippocampus and dentate gyrus. Amyloid β protein was not detected in cerebral cortex. We found similar patterns of amyloid β protein immunoreactivity in four F1 generation mice from both transgenic lines by four different amyloid β protein–specific rabbit polyclonal antibodies (*15*). Occasional clusters of amyloid β protein immunoreactivity were found in other regions of the CNS but not in a consistent pattern. Amyloid β protein immunoreactiv-

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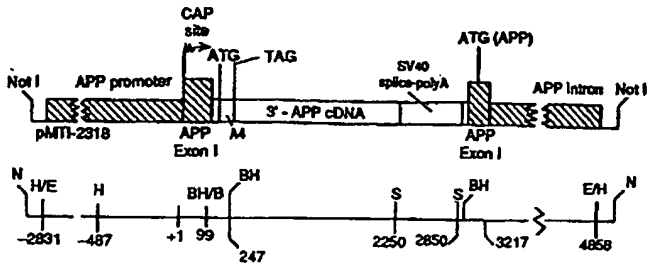

Fig. 1. Schematic representation of the ~7.7-kb Not I restriction fragment from pMTI-2318 (*18*) used to generate transgenic mouse lines AE101 and AE301 (*19*). A4, amyloid β protein; B, Bgl II; BH, Bam HI; E, Eco RI; H, Hind III; N, Not I; S, Sph I; BH/B, Bam HI–Bgl II fusion; and E/H or H/E, Hind III–Eco RI fusion.

ity was also associated with some but not all blood vessels in transgenic mice (Fig. 3).

Regions of hippocampus from transgenic and control mice were processed for electron microscopy (*16*). Ultrastructural correlates of the clustered dots of amyloid β protein immunoreactivity were present only in transgenic mice and consisted of intracellular accumulations of fibril-laden material (Fig. 4A) that resembled amyloid in brains from patients with AD. The cellular processes containing this amyloid-like material appeared distended, and some contained rough endoplasmic reticulum (Fig. 4B, arrows) and free ribosomes but not intermediate filament bundles (indicative of astrocytes), suggesting that they were dendrites. Amyloid-like deposits have not been detected in myelinated axons, although their presence in unmyelinated axons cannot be excluded. Ultrathin cryosections of transgenic mice hippocampus were also stained with antibodies to amyloid β protein and immunogold procedures. Gold particles in these electron micrographs were selectively enriched over the abnormal amyloid-like fibrils (*15*).

Our results illustrate that human amyloid β protein can accumulate in the CNS of transgenic mice and form amyloid-like profiles. This accumulation occurred despite low steady-state levels of amyloid β protein mRNA. Amyloid β protein deposits in postmortem brains from individuals with AD and DS are extracellular, whereas the amyloid β protein deposits in the 1-year-old transgenic mice are intracellular. Intracellular amyloid β protein immunoreactivity has also been observed in mouse hippocampal trisomy 16 grafts, a mouse model for DS (*17*). Although the source of extracellular amyloid β protein in AD is unknown, it is likely that at least a proportion of amyloid β protein has intracellular origins.

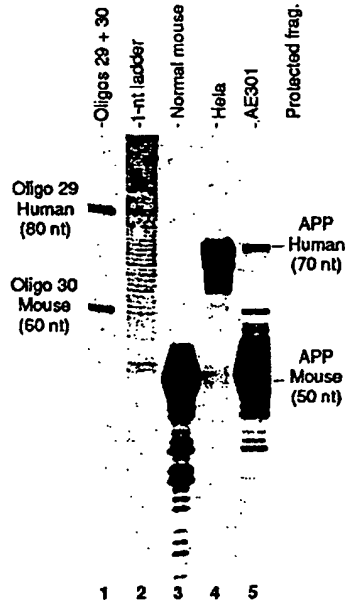

Fig. 2. S1 nuclease protection analysis of total RNA from normal and transgenic AE301 (F1) brain (*20*). The protected fragment sizes of the human (transgenic) and endogenous mouse APP oligonucleotide probes are 70 and 50 nucleotides (nt), respectively. Lane 1, human (oligo 29, 80 nt) and mouse (oligo 30, 60 nt) APP probes; lane 2, 1-nt DNA ladder; lane 3, normal mouse brain RNA; lane 4, Hela cell RNA; and lane 5, AE301 brain RNA. AE101, data not shown.

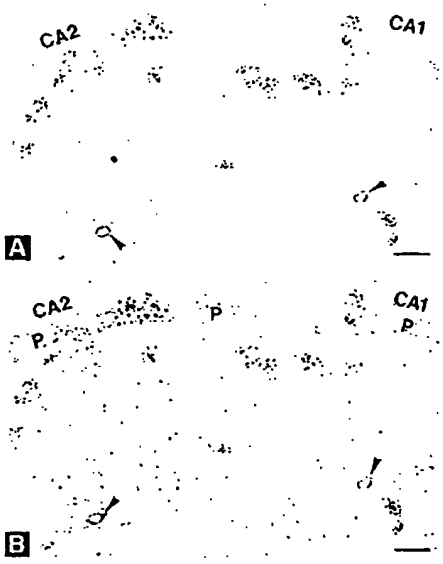

Fig. 3. Amyloid β protein distribution in a paraffin section from the hippocampus of a 1-year-old AE101 transgenic mouse that was photographed before (**A**) and after (**B**) hematoxylin counterstaining. Arrowheads, amyloid β protein deposits around blood vessels; and P, pyramidal cell layer of hippocampus. Scale bar: 200 μm.

Fig. 4. Electron micrographs of Epon sections from the hippocampal CA1 region of a 1-year-old AE101 transgenic mouse. (**A**) Amyloid-like deposits are densely stained by uranyl acetate and lead citrate (arrowheads). (**B**) Fibrils (arrowheads) and profiles of rough endoplasmic reticulum (arrows) are associated with amyloid-like deposits. Scale bars: (A) 1.0 μm; (B) 0.5 μm.

The APP promoter is active in most neurons in the mouse CNS (*13*). The accumulation of amyloid β protein in the transgenic mice, however, is restricted primarily to the hippocampus. These data raise the possibility that amyloid β protein expression alone is not sufficient to produce amyloid-like accumulations. Although amyloid β protein–positive processes have not yet been traced to cellular perikarya, it is likely that many are dendrites of neurons concentrated in hippocampal regions CA1 and CA2. The accumulation of amyloid β protein in the CNS of these transgenic mice appears to be developmentally regulated, as it is not significant before 6 months of age (*15*). The late onset of amyloid β protein accumulation suggests that either the steady-state concentration of amyloid β protein increases with age or that factors in addition to amyloid β protein expression participate in amyloid deposition.

Evidence of neuronal cell death, early signs of neuronal degeneration, or obvious signs of CNS dysfunction have not been detected in transgenic mice at 1 year of age. Older mice will be examined for behavioral and neuropathological changes associated with amyloid β protein accumulation. The introduction of gene constructs encoding native and mutant forms of APP into transgenic mice should allow elucidation of the cellular and molecular mechanisms involved in CNS amyloidosis.

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14. Brains from 1-year-old AE101 and AE301 transgenic and control mice were fixed with 4% paraformaldehyde. Paraffin sections of brains were cut (8 μm thick) and immunostained by rabbit polyclonal antiserum (90-29) to the amyloid β protein (1:500 dilution) and the avidin-biotin procedure (Vector Labs, Burlingame, CA). The pattern of amyloid β protein immunoreactivity in transgenic mouse brain sections was consistent when four different rabbit polyclonal antisera directed against amyloid β protein were used. Antisera 90-25 and 90-27 were directed against amino acids 1 to 28 of amyloid β protein; antisera 90-28 and 90-29 were directed against amino acids 1 to 42. All four rabbit polyclonal antisera exhibited intense immunoreactivity with senile plaques in sections of postmortem brain from AD patients (B. D. Trapp, unpublished data). Specificity of the immunoreactivity was established by the absence of immunoprecipitate in sections stained by amyloid β protein antiserum that had been absorbed with amyloid β protein and by Western blotting.
15. B. D. Trapp, unpublished data.
16. Brains from 1-year-old AE101 transgenic and control mice were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde. Ultrathin Epon sections of the hippocampus were cut, counterstained with uranyl acetate and lead citrate, and examined in a Hitachi H-600 electron microscope.
17. S.-J. Richards *et al.*, *EMBO J.* 10, 297 (1990).
18. The open reading frame for the 42-residue amyloid β protein (A4) was contained within a 148-bp Bgl II–Bam HI restriction fragment and was generated by site-directed mutagenesis [L. Kunkel *et al.*, *Methods Enzymol.* 154, 367 (1987)] of APP cDNA sequences with a synthetic oligonucleotide primer (5′-GGTGTTGTCATAGCGTAGGATCCGTCATCACCTTGGTG-3′). This Bgl II–Bam HI restriction fragment was ligated into the Bam HI site of pMTI-2307 [D. O. Wirak *et al.*, *EMBO J.* 10, 289 (1991)] to generate pMTI-2316. An ~2-kb Bam HI restriction fragment, containing APP 695 3′-end cDNA sequences, was inserted into the Bam HI site of pMTI-2316 to generate pMTI-2317. An 0.6-kb Sph I restriction fragment of pMTI-2304, containing SV40 RNA splicing signals [H. Okayama and P. Berg, *Mol. Cell. Biol.* 3, 280 (1983)] and SV40 polyadenylation signals (Bam HI–Bcl I restriction fragment from SV40 viral DNA) was ligated into a Sph I site of pMTI-2317 to generate PMTI-2318. The full-length cDNA encoding APP695 has been described [J. Kang *et al.*, *Nature* 325, 733 (1987)].
19. Transgenic mouse lines AE101 and AE301 were generated as described [D. Wirak *et al.*, *EMBO J.* 10, 289 (1991)]. In both transgenic mouse lines, multiple copies of the transgene have integrated as a head-to-tail tandem array (D. Wirak, unpublished data).
20. RNA was extracted from mouse brain and Hela cells as described [L. G. Davis, M.D. Dibner, J. F. Battey, *Basic Methods in Molecular Biology* (Elsevier, New York, 1986), pp. 130–135]. Synthetic oligonucleotides 29, 5′-GAGATAGAATACATTACTGATGTGTGGATTAATTCAAGTTCAGGCATCTACTTGTGTTACAGCACAGCTGGGCGTCCATA-3′, and 30, 5′-CGCGGGTGGGGCTTAGTTCTGCATTTGCTCAAAGAACTTGTAAGTTGGATAGGTTCCAAG-3′, were labeled with T4 polynucleotide kinase, and their specific activities were 6.04×10^8 and 5.72×10^8 cpm/μg, respectively. S1 nuclease protection analysis [S. Sisodia *et al.*, *Nucleic Acids Res.* 15, 1999 (1987)] was performed with total RNA (50 μg per sample) and 1×10^6 cpm of each ^{32}P-labeled oligonucleotide.
21. We thank C. Kundel and A. Lee for assistance in mini-gene constructions; S. Rocks and T. Garrison for transgenic animal colony management; G. Davis, T. Buckholz, and P. Tamburini for the synthesis and purification of amyloid β proteins; M. Broggi for assistance in polyclonal antibody production; P. Rae and P. Talalay for reviewing the manuscript; R. Graham for editing and typing the manuscript; C. Cootauco and E. Mulrenin for assistance in performing the morphological studies; and K.-H. Büchel for his support.

30 April 1991; accepted 20 June 1991

Evidence for the Effects of a Superantigen in Rheumatoid Arthritis

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While studying the αβ T cell receptor repertoire in rheumatoid arthritis (RA) patients, we found that the frequency of $V_\beta14^+$ T cells was significantly higher in the synovial fluid of affected joints than in the peripheral blood. In fact, $V_\beta14^+$ T cells were virtually undetectable in the peripheral blood of a majority of these RA patients. β-chain sequences indicated that one or a few clones dominated the $V_\beta14^+$ population in the synovial fluid of individual RA patients, whereas oligoclonality was less marked for other Vβ's and for $V_\beta14$ in other types of inflammatory arthritis. These results implicate $V_\beta14$-bearing T cells in the pathology of RA. They also suggest that the etiology of RA may involve initial activation of $V_\beta14^+$ T cells by a $V_\beta14$-specific superantigen with subsequent recruitment of a few activated autoreactive $V_\beta14^+$ T cell clones to the joints while the majority of other $V_\beta14^+$ T cells disappear.

RA IS AN AUTOIMMUNE DISEASE characterized by long-term inflammation of multiple joints. Mononuclear cell infiltration of the synovial membrane eventually can lead to the destruction of articular cartilage and surrounding structures. Because of its high frequency and potentially severe nature, this disease is a major cause of long-term disability in adults. Although the pathogenesis of RA and other similar autoimmune diseases remains unknown, genetic and environmental factors have been implicated. Several lines of evidence suggest that T cells specific for self-antigens may play a critical role in the initiation of these diseases. In the case of RA, the linkage of the disease to the DR4 and DR1 alleles of the class II genes of the major histocompatibility complex (MHC) and the finding of sometimes oligoclonal, activated $CD4^+$ T cells in synovial fluid and tissue of affected joints (*1*, *2*) suggest the involvement of $CD4^+$, αβ T cell receptor (TCR)–bearing, class II–restricted T cells in the disease. This view is supported by the finding that partial elimination or inhibition of T cells by a variety of techniques can lead to an amelioration of disease in certain patients (*3*).

Usually, potentially autoreactive T cells are deleted or inactivated by encounter with self-antigen during their development, before they can damage the individual (*4*, *5*). To understand autoimmunity one must therefore understand how self-reactive T cells escape these processes to become part of the mature T cell pool and what factors control whether these cells will remain quiescent or become activated to induce autoimmune disease. It is possible that a self-

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AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE

SCIENCE

ISSN 0036-8075
5 JULY 1991
VOLUME 253
NUMBER 5015

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Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10857–10861, November 1992
Neurobiology

Deposition of β/A4 immunoreactivity and neuronal pathology in transgenic mice expressing the carboxyl-terminal fragment of the Alzheimer amyloid precursor in the brain

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Communicated by Richard F. Thompson, July 7, 1992

ABSTRACT **The deposition of amyloid in senile plaques and along the walls of the cerebral vasculature is a characteristic feature of Alzheimer disease. The peptide comprising the carboxyl-terminal 100 amino acids of the β-amyloid precursor protein (βAPP) has been shown to aggregate into amyloid-like fibrils *in vitro* and to be neurotoxic, suggesting that this fragment may play a role in the etiology of Alzheimer disease. To address this question, we expressed this carboxyl-terminal 100-amino acid peptide of βAPP in transgenic mice under the control of the brain dystrophin promoter. We used an antibody to the principal component of amyloid, β/A4, to demonstrate cell-body and neuropil accumulation of β/A4 immunoreactivity in the brains of 4- and 6-month-old transgenic mice. Only light cytoplasmic staining with this antibody was visible in control mice. In addition, immunocytochemical analysis of the brains with an antibody to the carboxyl terminus of βAPP revealed abnormal aggregation of this epitope of βAPP within vesicular structures in the cytoplasm and in abnormal-appearing neurites in the CA2/3 region of the hippocampus in transgenic mice, similar to its aggregation in the cells of Alzheimer disease brains. Thioflavin S histochemistry suggested accumulations of amyloid in the cerebrovasculature of transgenic mice with the highest expression of the βAPP-C100 transgene. These observations suggest that expression of abnormal carboxyl-terminal subfragments of βAPP *in vivo* may cause amyloidogenesis and specific neuropathology.**

Alzheimer disease (AD) is a neurodegenerative disorder characterized by progressive loss of memory and declining cognitive function beginning in late life. A prominent feature of AD neuropathology is the deposition of amyloid in senile plaques and along the walls of the cerebrovasculature. The peptide fragment termed β/A4 (39–43 amino acids; refs. 1 and 2) is the principal constituent of the amyloid deposits, although plaques contain numerous other components. The β-amyloid protein precursor (βAPP), from which β/A4 is derived, is a transmembrane protein in which the β/A4 peptide spans the border between the extracellular domain and the transmembrane region (3, 4). Normal cleavage of βAPP in the secretory pathway occurs at the β/A4 Lys^{16}-Leu^{17} peptide bond (5). Recent data, however, have revealed that multiple β/A4-containing carboxyl-terminal fragments of βAPP are also produced in the brain, via the endosomal–lysosomal system (6).

Additional evidence has implicated at least one of these fragments, the carboxyl-terminal 100 amino acids of βAPP, in the development of AD neuropathology. This fragment, which spans the β/A4 and cytoplasmic domains, is amyloidogenic (7–9) and neurotoxic both *in vitro* (10, 11) and *in vivo* (12). To test the hypothesis that this neurotoxic fragment may play a role both in amyloidogenesis and in the development of the progressive neuropathology of AD, we introduced into mice a transgene carrying the sequence for the carboxyl-terminal 104 amino acids of βAPP (βAPP-C104) under control of the brain dystrophin promoter (13). Both founders and F_1 transgenic progeny manifested (*i*) accumulation of β/A4 immunoreactive material in neuronal cell bodies and (*ii*) a subcellular shift of immunoreactivity for the carboxyl terminus of βAPP from its normal distribution throughout the cell soma to aggregates in the cytoplasm and the neuropil in the hippocampus. Mice in three of the nine lines examined, with the most robust transgene expression in the brain, displayed accumulation of β/A4 immunoreactivity in abnormal-appearing neurites, as well as thioflavin S-fluorescent deposits in the cerebrovasculature. These neuropathological features were not seen in control mice.

METHODS

Production of Transgenic Mice. The *Bgl* II–*Sma* I fragment of the βAPP-695 cDNA [base pairs (bp) 1769–2959] was cloned into a modified form of plasmid pRSV in which the Rous sarcoma virus promoter was replaced with the dystrophin neural promoter (13). The βAPP-C104 transgene, together with the upstream dystrophin promoter and the downstream simian virus 40 splice and polyadenylylation sequences, was excised from plasmid sequences by digestion with *Mlu* I and *Bam*HI and was microinjected into the pronuclei of fertilized eggs from F_2 hybrid mice (C57BL/6 × SJL) at the National Transgenic Development Facility (DNX, Princeton, NJ). The injected mouse eggs were reimplanted into pseudopregnant recipient females.

Immunochemical and Histologic Analysis of Transgenic and Control Mice. Fourteen F_1 backcross progeny from six different founder lines (ages, 3.5–4 months) and founders only from three additional lines (ages, 6 months) were analyzed histologically. We also analyzed 8 age-matched C57BL/6 and SJL controls. All 22 mice were subjected to each immunocytochemical and histological analysis.

E1-42 immunocytochemistry on mouse and human sections and F5 immunocytochemistry on human sections were carried out as described by Cummings *et al.* (14). F5 and F8 immunocytochemistry on mouse brains was carried out as described by Neve *et al.* (12). All comparisons of transgenic and control mice were made using tissue processed in parallel and developed with diaminobenzidine for equivalent periods

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Abbreviations: AD, Alzheimer disease; βAPP, β-amyloid protein precursor.

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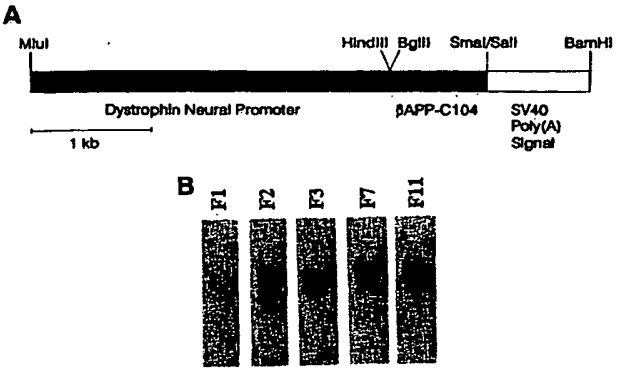

FIG. 1. (*A*) Schematic of the brain dystrophin promoter–βAPP-C104 fusion transgene. SV40, simian virus 40. (*B*) Southern blot analysis of selected transgenic lines.

of time. Sections incubated in parallel without E1-42, F5, or F8 primary antibody failed to develop any staining.

Sections subjected to thioflavin S histochemistry were incubated for 20 min in 1:1 absolute ethanol/chloroform and then rinsed three times for 1 min in 95% ethanol, 3 min in 70% ethanol, 3 min in 50% ethanol, and 3 min in water. Sections were then incubated for 4 min with 1% (wt/vol) thioflavin S (Sigma) in water and differentiated in 80% ethanol.

RESULTS

Characterization of Transgenic Mice Carrying the βAPP-C104 cDNA Under Control of the Dystrophin Brain Promoter. We anticipated that the low level of transcripts controlled by the dystrophin brain promoter might be important in allowing survival of βAPP-C104 transgenic mice beyond the embryonic stage. Hence, a 4.65-kilobase (kb) DNA fragment containing a dystrophin brain promoter–βAPP-C104 fusion gene with the simian virus 40 early-region splice and polyadenylylation sequences (Fig. 1*A*) was isolated and microinjected into the male pronuclei of fertilized eggs from F_2 hybrid mice

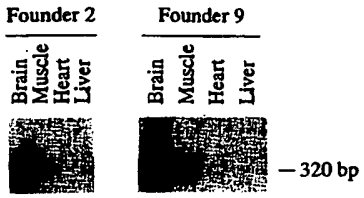

FIG. 2. Predominant brain expression of the βAPP-C104 transgene is revealed by reverse transcription–PCR. Hybridization of a radiolabeled interior oligonucleotide to a Southern blot of the amplification products from four tissue RNAs in two of the transgenic lines is shown.

(C57BL/6 × SJL). PCR analysis of tail DNA from 36 offspring revealed that 12 of the mice were positive for the transgene; 10 survived for further analysis. We used Southern blot analysis to estimate the transgene copy number in *Bam*HI-digested DNA from each founder line (representative lanes are shown in Fig. 1*B*). Positively hybridizing bands exceeded 20 kb in all founder lines, indicating incorporation of the transgene into the mouse genome (*Bam*HI does not cleave within the transgene). Copy number of the transgene ranged from 1 (line 4) to >20 (lines 2, 7, and 11). Seven founder mice produced transgenic offspring in crosses with normal C57BL/6 mice, and Southern blot analysis of selected F_1 and F_2 progeny showed that the transgenic DNA was inherited with no observable rearrangements or changes in copy number (data not shown).

Expression of the βAPP-C104 Transgene Predominantly in the Brain. We analyzed RNA from brain, skeletal muscle, heart, and liver of F_1 transgenic progeny from six founders at the age of 4 months and from the three founder mice that did not produce offspring when they reached the age of 6 months. Reverse transcription was coupled with PCR as described (15) to amplify a 320-bp segment of the transgene RNA (Fig. 2) and revealed that predominant expression of the transgene occurred in the brain. Highest expression was seen in lines 2, 3, 4, and 7. Although the transgene was transcribed at low levels in other tissues in some of the lines, in all transgenic

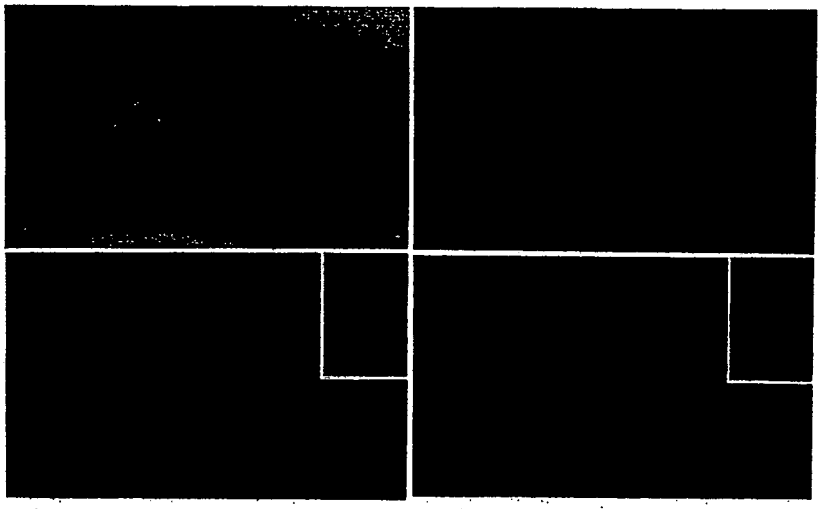

FIG. 3. (*A* and *B*) E1-42 immunoreactivity in the hippocampus of a transgenic mouse (*A*), compared with that of a control mouse (*B*). While low-level staining of cell bodies in the pyramidal cell layer and in additional scattered cells is seen in the control mouse (*B*), darker punctate accumulations of E1-42 immunoreactivity in the pyramidal cell layer (*A*, arrow) and throughout the hippocampus (*A*) are unique to the transgenic mice. Higher magnifications of the punctate intracellular deposits of E1-42 immunoreactivity in transgenic animals are shown in Fig. 4. (*C* and *D*) E1-42 immunoreactivity in the parietal cortex of a transgenic mouse (*C*) and a control mouse (*D*). Differences in E1-42 immunoreactivity between transgenic and control mice in the parietal cortex are much less pronounced than in the hippocampus. Preabsorption of the E1-42 antibody with 30 μg of peptide per μl of antibody resulted in absence of staining (*Insets*). (Bars = 100 μm.)

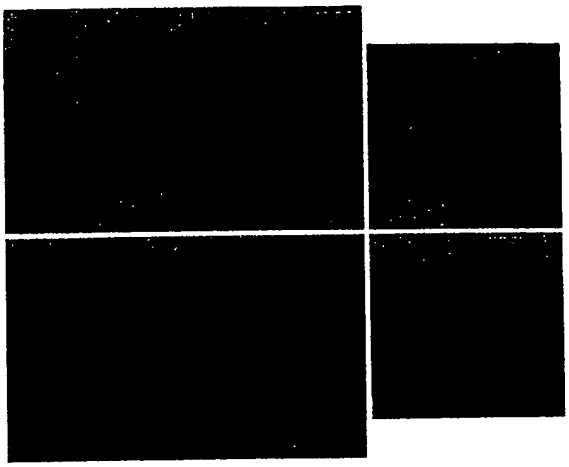

FIG. 4. High-power depictions of the punctate intracellular deposits of E1-42 immunoreactivity in transgenic mouse (*A* and *C*), and comparison with control (*B* and *D*). Note the darkly staining intracellular aggregates of E1-42 immunoreactivity in the hippocampus of transgenic mouse (*A*, arrowheads); increased magnification of one of these aggregates is shown in *C*. In contrast, E1-42 immunoreactivity appears as pale homogeneous cytoplasmic staining in some hippocampal neurons (*B*, arrowheads); a more-strongly stained neuron is shown at higher power in *D*. Note the lack of punctate accumulations of E1-42 immunoreactivity in these cells. (Bars = 10 μm.)

animals its expression was ≥10 times higher in brain than in any other tissue examined.

Analysis of β/A4 Immunoreactivity in the Brains of the Transgenic Mice. We used an affinity-purified polyclonal antibody, E1-42, raised against a peptide representing the 42-amino acid β/A4 fragment, to detect β/A4 epitopes in the transgenic mouse brains. This antibody does not immunostain normal βAPP in the human brain but is specific for amyloid cores in the neuritic plaques, as well as diffuse amyloid deposits that are not detectable by conventional histological stains for amyloid, such as thioflavin S (14). Immunostaining of control mouse brains with E1-42 was slightly different from that of control human brains, in that it showed very pale homogeneous staining of cell bodies in all nontransgenic mice (Fig. 3*B*; Fig. 4 *B* and *D*). In contrast, immunostaining of the brains of 4-month-old F_1 transgenic mice from six lines, and of three 6-month-old founder mice, revealed abnormal intraneuronal β/A4 immunoreactivity throughout the brain in all of the transgenic mice. The strongest E1-42 immunopositive cells, however, predominated in the hippocampus [Fig. 3*A*; compare with E1-42 immunoreactivity in the parietal cortex, which is very similar between transgenic (Fig. 3*C*) and control (Fig. 3*D*) animals]. Preabsorption of the E1-42 antibody with the β/A4 peptide resulted in loss of specific staining (*Insets*, Fig. 3 *C* and *D*). In most cases, the E1-42 immunoreactivity occurred as punctate deposits within neurons that had a rounded, compact appearance (Fig. 4 *A* and *C*). The intracellular accumulati n of β/A4 immunoreactivity was particularly prominent within the hilus (Fig. 5), and close examination of the E1-42 staining in this region showed that the immunoreactivity extended beyond the cell body, with punctate deposits visible in some processes (Fig. 5*B*). Deposits of β/A4 immunoreactivity were not visible in the hilus of control animals (Fig. 5*C*) and did not resemble the age-associated inclusions in normal and transgenic C57BL/6J mice that are occasional immunocytochemical artifacts (16).

Transgenic mice of lines 2, 3, and 7, with the most robust transgene expression in the brain, displayed punctate accumulations of β/A4 immunoreactivity in sh rt, somewhat

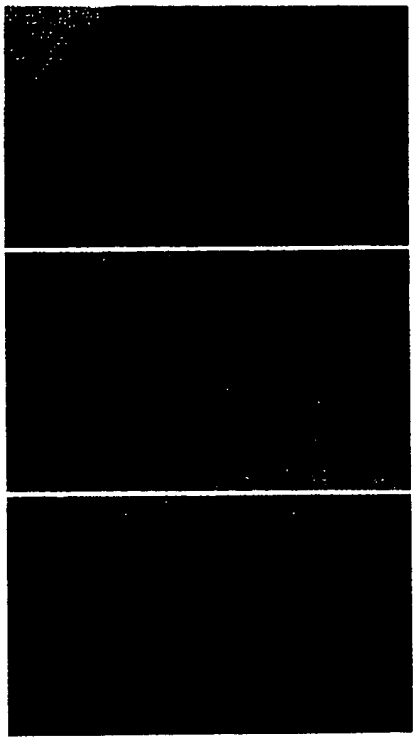

FIG. 5. E1-42 immunoreactivity in the hilus of a transgenic mouse (*A* and *B*), compared with that of a control mouse (*C*). This region of the hippocampus possessed the highest density of cells with intracellular β/A4 immunoreactive deposits (*A*); punctate deposits are also visible in the neuropil (*B*). An enlargement of the field outlined in *A* is depicted in *B*. A comparable enlargement of the hilar field of a control animal is depicted in *C*. (Bars = 100 μm.)

curly, abnormal-appearing fibers that were apparent in the stratum radiatum of the CA2/3 region of the hippocampus (Fig. 6), similar to the accumulations shown in Fig. 5 in the hilus. The emergence of E1-42 immunoreactivity in the neuropil may represent a later stage of amyloid deposition or pathology than that seen in the cell soma, since it was

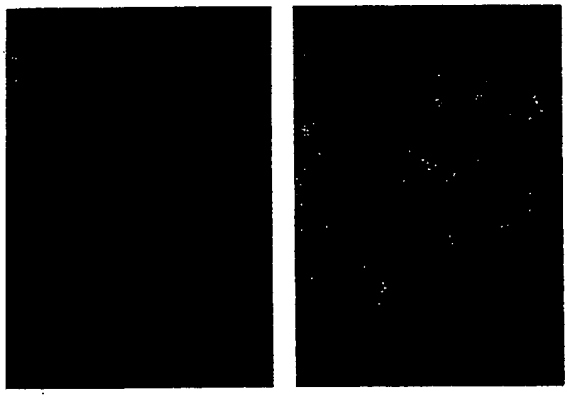

FIG. 6. E1-42 immunoreactivity in the neuropil at the CA2/3 boundary in a transgenic mouse with a high copy number of the transgene. Punctate β/A4 deposits have virtually disappeared from cell bodies of this region in this mouse and are instead found accumulated in curly dystrophic-appearing fibers throughout the area (*A*). A greater magnification of a sector of the region displayed in *A* is shown in *B*. (Bar in *A* = 100 μm; bar in *B* = 10 μm.)

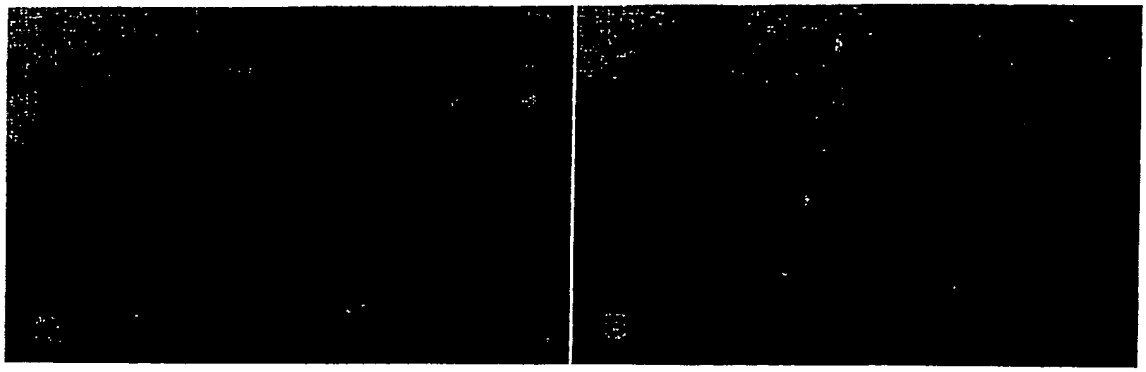

FIG. 7. F5 immunostaining of transgenic (*A*) and control (*B*) animals at the CA2/3 boundary in the hippocampus. Note that the F5 epitope (the carboxyl terminus of βAPP) has accumulated in punctate vesicular structures within the cell somata and has collected in the neuropil in the transgenic animal. (Bars = 10 μm.)

confined to those mouse lines with the highest transgene expression and was never seen in controls.

Subcellular Shift of the βAPP Carboxyl Terminus. Previous work showed that immunoreactivity for the carboxyl-terminal epitope of βAPP segregated into enlarged intracellular organell s in the hippocampus in AD (17) and highlighted neuropil aberrations in the hippocampus of mice transplanted with transfected PC12 cells expressing βAPP-C104 (12). We detected a similar phenomenon in the transgenic mice. Staining of the brain sections with F5, an antibody to the carboxyl-terminal 9 amino acids of βAPP (17), showed a striking change in the subcellular localization of the F5 epitope, which was particularly evident in the CA2/3 region of the hippocampus in transgenic mice. Whereas control mice displayed homogeneous F5 staining predominantly of the neuronal somata in this region (Fig. 7*B*), the F5 immunoreactivity in the transgenic mice took on a punctate appearance in the cell somata (Fig. 7*A*). This punctate staining, which often extended markedly into the neuronal processes, was also revealed with an independent antibody to the carboxyl terminus of βAPP (F8; data not shown). Adjacent Nissl-stained sections did not reveal detectable gross morphological abnormalities in the area of altered F5 staining, suggesting that the intracellular disorganization of the F5 epitope does not reflect cell body degeneration in transgenic mice of this age.

In mice from lines 2, 3, and 7, with highest transgene expression in the brain, the cells in the hippocampal region showed particularly dense reaction product in the neuropil, and the F5 reactivity in the soma took the form of larger accumulations, as if the punctate vesicular immunoreactiv material was fusing or aggregating (Fig. 8*A*). The distribution of the F5 immunoreactivity in these cells was very similar to its distribution in CA1 hippocampal neurons in human AD brains (Fig. 8*C* and ref. 17) relative to controls (Fig. 8*D*). The similarity in the appearance of the punctate cell-body F5 immunoreactivity in transgenic animals and in AD individuals in the hippocampus is marked (Fig. 8 *A* and *C*), and is suggestive of an early stage of pathology.

Cerebrovascular Staining of Transgenic Mice with Thioflavin S. The mice from lines 2, 3, and 7, with the highest brain expression of the transgene, displayed thioflavin S fluorescence associated with blood vessels (Fig. 9), which we also observed in AD brains stained with thioflavin S. This fluorescence suggested that amyloid had accumulated in or around the cerebral blood vessels of these transgenic animals.

DISCUSSION

We have shown that expression of βAPP-C104 in the brains of transgenic mice can lead to the deposition of intracellular

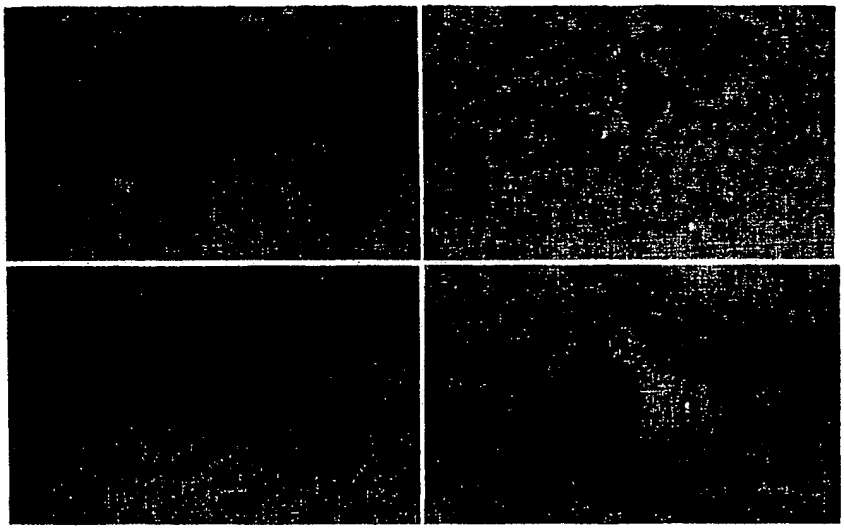

FIG. 8. (*A* and *C*) Cytoplasmic accumulations of the F5 epitope in hippocampal pyramidal cells of a transgenic mouse and a human AD brain, respectively. (*B* and *D*) F5 immunostaining of hippocampal pyramidal cells in a control mouse and a control human brain, respectively. (Bars = 10 μm.)

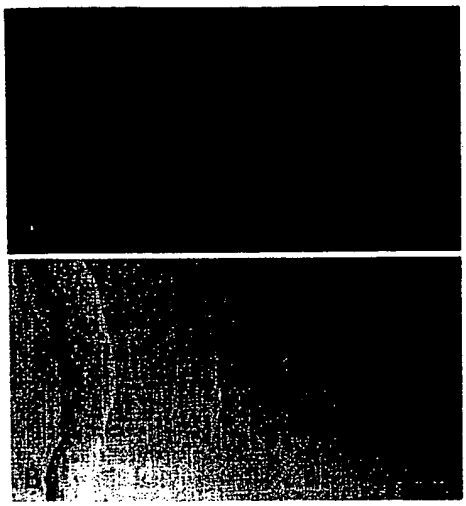

FIG. 9. (*A*) Thioflavin S fluorescence around blood vessels in the hippocampus of a transgenic mouse from line 2. (*B*) Thioflavin S-positive structures correlate with blood vessels as shown with Nomarsky optics (arrowheads). (Bars = 100 μm.)

β/A4 immunoreactivity and cerebrovascular amyloid, as well as early neuronal pathology as manifested by abnormal intracellular accumulations of the carboxyl-terminal epitope of βAPP, some of which occurred in dystrophic-appearing neurites. Interestingly, Quon *et al.* (18) also detected abnormal neurites with punctate accumulations of β/A4 immunoreactivity in transgenic mice overexpressing βAPP-751.

Previous work has shown that the enlarged intracellular organelles into which the F5 immunoreactivity segregates in AD, similar to those we observed in the transgenic mice, are probably fused lysosomes (17). The dense F5 immunostaining in these enlarged organelles was particularly prominent in regions of the hippocampus that were heavily invested with pathology, such as CA1, in which the heavy staining of the pyramidal cells was accompanied by atrophy of many of these cells (18). Although Nissl and Bielschowsky stains did not reveal gross neuronal death in any of the transgenic brains, it is possible that accumulation of the F5 epitope in enlarged lysosomes may presage neuronal degeneration.

We do not know whether the F5 immunoreactivity that aggregates into cellular compartments in the transgenic mice reflects the presence of only the carboxyl terminus of βAPP in the swollen organelles or also the larger precursor protein. An antibody to protease nexin II (the extracellular secreted portion of βAPP) detects enlarged punctate accumulations of βAPP within degenerating neurons and dystrophic neurites, as well as within plaques, in AD brain (14). These observations suggest that the compartmentalization of βAPP, presumably into lysosomes, during the disease process is not limited to the carboxyl terminus of the molecule. These data, coupled with those of Cataldo and Nixon (19), Joachim *et al.* (20), and Golde *et al.* (6), also imply that the generation of amyloid deposits is not exclusively an extracellular process and may in fact begin within the neuron.

Thioflavin S-positive material was observed in the cerebrovasculature of three of the transgenic animals. This material was not immunoreactive with E1-42, which is consistent with the failure of this antibody to detect thioflavin S-positive cerebrovascular amyloid in AD brain. During the analyses of the βAPP-C104 transgenic mice, we tested five additional β/A4 antibodies, all of which stained plaque amyl id in AD brain and punctate intracellular deposits in the transgenic mice with ut staining cerebr vascular amyl id in either AD brain or the mouse brains. Recent data (21) indicate that there are conformati nal differenc s in β/A4 derived from plaque and cerebrovascular amyloid, respectively. Moreover, thioflavin S is a stain that defin s th structure termed amyloid, which includes the deposits found in various peripheral amyloid ses as w ll as those detected in cerebral amyl ideses (22). Whil β/A4 is the principal compon nt of amyloid in AD, other mol cules contribut in varying degrees t the f rmation of this thioflavin S-positive structure and may constitute a relatively large percentage of this structure in the cerebrovasculature of the transgenic mice.

These βAPP-C104 mice may provide a model for dissecting the molecular events that lead to the accumulation of amyloid and possibly neurodegeneration in AD brain and may be useful for developing therapeutic agents that can halt or reverse these processes.

We appreciate the many helpful discussions we had with Drs. K. Kosik and R. Nixon. We thank Dr. P. Brandt for computer help with the figures, Dr. K. Ivins for help with perfusing the mice, Dr. D. Schenk for the F5 and F8 antibodies, Dr. M. Rosenberg for the simplified protocol for extraction of tail DNA, and J. Zimmerman for making human AD and age-matched control tissue available to us. We acknowledge National Institute of Child Health and Human Development Contract NO1-HD-0-2911 in support of DNX, Inc., as the source of the transgenic mice. This research was supported by Contract 90-00198 from the California State Department of Health Services, a Focused Giving grant from Johnson & Johnson, a Metropolitan Life Foundation Award, and National Institutes of Health Grants HD18658 and NS28406 (R.L.N.). F.M.B. is the Betty Banker Fellow of the Muscular Dystrophy Association.

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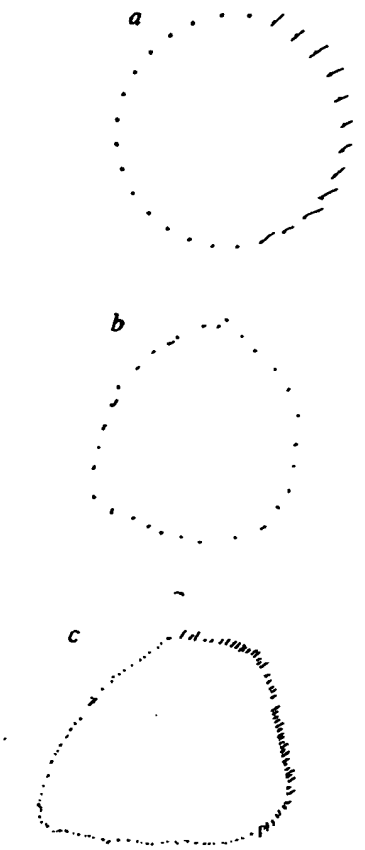

FIG. 4 J.R.'s attempts to cancel the local components of a predrawn figure (*a*); to reproduce the figure (*b*); and to draw the figure and then cancel its local components (*c*).

even when focal attention cannot be automatically directed to local components on the left of a global figure. J.R.'s neglect is thus neither purely perceptual nor purely motor; it rather arises from an input-deficit to a 'premotor' mechanism[10] committed to the exogenous control of focal attention. Lesions that 'disrupt the cross-talk between inferior posterior areas' can reduce or eliminate interactions between global and local processing[9]. Whether this disruption in J.R. is due solely to her right hemisphere lesion (or also implicates her left thalamic infarct) is unclear. One further problem remains: why is J.R. unaware of the discrepancy between her global percepts and local actions? The process of local completion can mask perceptual incongruity but cannot then control appropriate manual output. J.R. can perceive the whole forest but cannot use that percept to search for and cut down the trees on the left thereof. □

Received 6 September; accepted 12 December 1994.

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ACKNOWLEDGEMENTS. This work was supported by the Medical Research Council.

Alzh im r-typ neuropathology In transg nic mice ov rexpr ssing V717F β-amyloid pr curs r pr t in

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ALZHEIMER'S disease (AD) is the most common cause of progressive intellectual failure in aged humans. AD brains contain numerous amyloid plaques surrounded by dystrophic neurites, and show profound synaptic loss, neurofibrillary tangle formation and gliosis. The amyloid plaques are composed of amyloid β-peptide (Aβ), a 40–42-amino-acid fragment of the β-amyloid precursor protein (APP)[1]. A primary pathogenic role for APP/Aβ is suggested by missense mutations in APP that are tightly linked to autosomal dominant forms of AD[2,3]. A major obstacle to elucidating and treating AD has been the lack of an animal model. Animals transgenic for APP have previously failed to show extensive AD-type neuropathology[4–10], but we now report the production of transgenic mice that express high levels of human mutant APP (with valine at residue 717 substituted by phenylalanine) and which progressively develop many of the pathological hallmarks of AD, including numerous extracellular thioflavin S-positive Aβ deposits, neuritic plaques, synaptic loss, astrocytosis and microgliosis. These mice support a primary role for APP/Aβ in the genesis of AD and could provide a preclinical model for testing therapeutic drugs.

Transgenic mice were generated using a platelet-derived growth factor (PDGF)-β promoter[11] driving a human APP (hAPP) minigene encoding the $APP_{717V\rightarrow F}$ mutation associated with familial AD[12] (PD-APP; Fig. 1*a*). The construct contained APP introns 6–8, allowing alternative splicing of exons 7 and 8.

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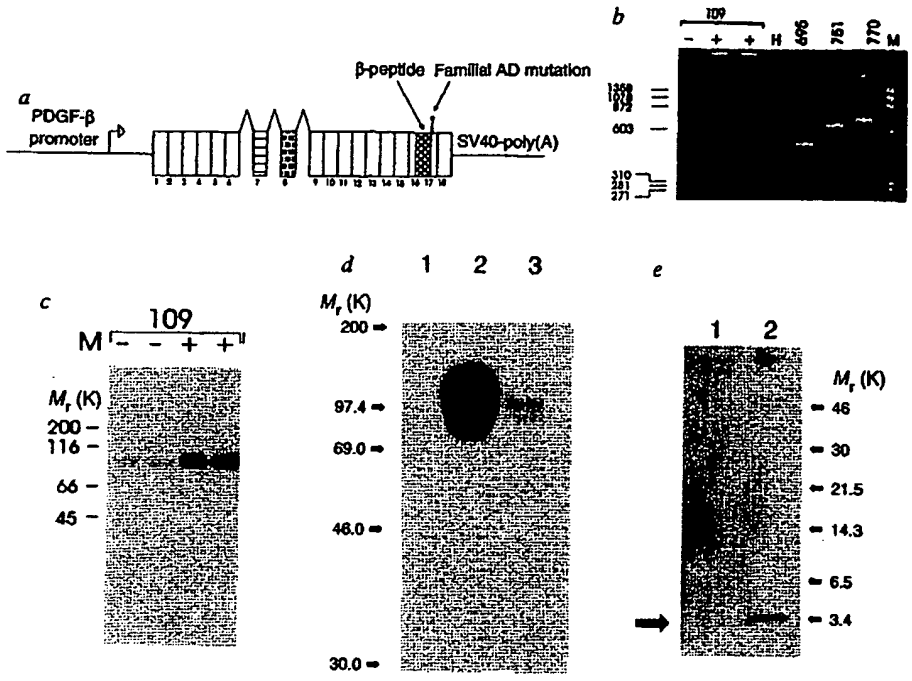

Fig. 1 analysis of app expression in brain tissue of pd-app transgenic mice. *a*, Map of the APP construct (PD-APP) minigene used to generate transgenic mice (not to scale). The construct contains the PDGF β-chain promoter, full-length hAPP cDNA encoding the Val-to-Phe mutation at codon 717, and the inclusion of genomic sequences for hAPP introns 6-8. *b*, RT-PCR analysis demonstrates the presence of transcripts encoding the 695, 751 and 770 isoforms of hAPP in transgenic animal brains (+) but not in brains from non-transgenic littermates (−). Control reactions using human brain RNA (H) as well as cDNA clones encoding hAPP 695, 751 or 770 are also shown. M, Markers (calibration in bp). *c*, Immunoblot analysis of total APP expression (human and mouse) in transgenic mouse (+) and control littermate (−) brain tissue using a C-terminal APP antibody, α6. *d*, Human-specific APP expression in brain tissue from a 6-month-old non-transgenic littermate (lane 1), transgenic mouse (lane 2) or human AD cortex (lane 3) using immunoblotting with the human-specific APP antibody 8E5. *e*, Immunoprecipitation and immunoblot analysis of Aβ from the brain tissue of a 9-month-old non-transgenic littermate (lane 1) and a 9-month-old transgenic mouse (lane 2).

METHODS. The PDGF β-chain 5′ flanking sequence included 1.3 kb upstream of the transcription initiation site and ~70 bp of 5′ untranslated region, ending at the *Aur*II site[20]. The β-APP sequences were derived from human β-APP cDNA starting with the *Nru*I site in exon 1 through to the end of exon 6 and from the beginning of exon 9 through to exon 18, including the 3′ untranslated sequence ending at the *Sph*I site, and from human genomic sequences from exons 6–9, including all intervening introns. To introduce the familial AD mutation at position 717, Val was mutated to Phe (G to T, using the P-Select mutagenesis kit; Promega). The late SV40 polyadenylation signal was provided by the 240-bp *Bam*HI to *Bcl*I fragment. Plasmid sequences (pUC) were removed by *Sac*1 and *Not*1 digestion before microinjection. Transgenic mice were generated using standard techniques[19], except that PD-APP DNA was microinjected into the embryos at the two-cell stage. Seven founder mice were generated and line 109 was used for extensive analysis. RNA was isolated from brain tissue as described[20] and subjected to RT-PCR as described[21] using human-specific APP primers (5′-CCGATGATGACGAGGACGAT-3′, 5′-TGAACACGTGACGAGGCCGA-3′) with the following PCR conditions: 40 cycles of 1 min at 94 °C, 40 s at 60 °C, 50 s at 72 °C. The identities of the human APP RT-PCR bands from the transgenic mouse RNA were verified by subcloning and sequencing. Analysis of holo-APP involved brain homogenization in 10 volumes of PBS containing 0.5 mM EDTA, 10 μg ml^{-1} leupeptin and 1 mM PMSF. Samples were spun at 12,000*g* for 10 min and the pellets resuspended in RIPA (150 mM NaCl, 50 mM Tris, pH 8.0, 20 mM EDTA, 1.0% deoxycholate, 1.0% Triton X-100, 0.1% SDS, 1 mM PMSF and 10 μg ml^{-1} leupeptin (*d*)). Samples (each containing 30 μg total protein) were analysed by SDS–PAGE, transferred to Immobilon and reacted with either the holo-APP antibody, α6 (ref. 22), or 8E5 monoclonal antibody. 8E5 was prepared against a bacterial fusion protein encompassing hAPP residues 444–592 (ref. 22) and is human-specific showing essentially no crossreactivity against mouse APP (*d*, lane 1). For immunoblot analysis of Aβ (*e*), a 9-month-old mouse brain was homogenized in 5 ml 6 M guanidine HCl, 50 mM Tris, pH 7.5. The homogenate was centrifuged at 100,000*g* for 15 min and the supernatant was dialysed against H_2O overnight adjusted to PBS with 1 mM PMSF and 25 μg ml^{-1} leupeptin. This material was immunoprecipated with antibody 266 resin, and immunoblotted with the human-specific Aβ antibody, 6C6, as described[13].

We used only heterozygous animals. Southern analysis of 104 from four generations showed that ~40 copies of the transgene were inserted at a single site and transmitted in a stable manner (data not shown). Human APP messenger RNA was produced in several tissues of the transgenic mouse, but at especially high levels in brain (not shown). RNase protection assays revealed ~18-fold more APP expression in the brains of line 109 animals than in most previously described lines expressing neuron-specific enolase (NSE)-promoter-driven APP transgenes (not shown; refs 4, 8–10). In addition, the three major splicing variants of hAPP mRNA (695, 751, 770)[1] were expressed in the transgenic mice, as evidenced by reverse-transcriptase polymerase chain reaction (RT-PCR) (Fig. 1*b*). Immunoblot analysis of brain homogenates using either a holo-APP polyclonal antibody or a human-specific APP monoclonal antibody revealed hAPP overexpression in the transgenic mouse at levels ≥10-fold higher than either endogenous mouse APP levels (Fig. 1*c*, *d*) or those in AD brain (Fig. 1*d*). Using human-specific Aβ antibodies, we isolated a 4K Aβ-immunoreactive peptide from the brains of the transgenic animals, which corresponds to the relative molecular mass of Aβ (Fig. 1*e*). Brain levels of Aβ were at least 10-fold higher in line-109 animals than in the previously described hAPP transgenic mice (not shown; ref. 9). Finally, embryonic day-16 cortical cell cultures from transgenic animals constitutively secreted human Aβ, including a substantial fraction of Aβ 1–42 (5 ng ml^{-1} total Aβ; 0.7 ng ml^{-1} Aβ 1–42), as detected in media by human-specific Aβ enzyme-linked immunosorbent assays (not shown; refs 9 and 13). Thus, line-109 animals greatly overexpressed human APP mRNA, holo-APP and Aβ in their brains.

Brains from 18 transgenic animals and 12 age-matched non-transgenic littermate controls (4–13 months old) representing three generations of the line-109 pedigree were extensively examined histopathologically. Between 4–6 months of age (n=7), no obvious pathology was detected; however, at ~6–9 months of age (n=7), transgenic animals began to exhibit deposits of human Aβ in the hippocampus, corpus callosum and cerebral cortex, but not in other brain regions. These increased with age, and by eight months many deposits (30–200 μm) were seen (Fig. 2*a*). As the animals aged (≥9 months; n=4), the density of the plaques increased (Fig. 2*c*) until the Aβ-staining pattern resembled that of AD (Fig. 2*a*, inset). Robust pathology was

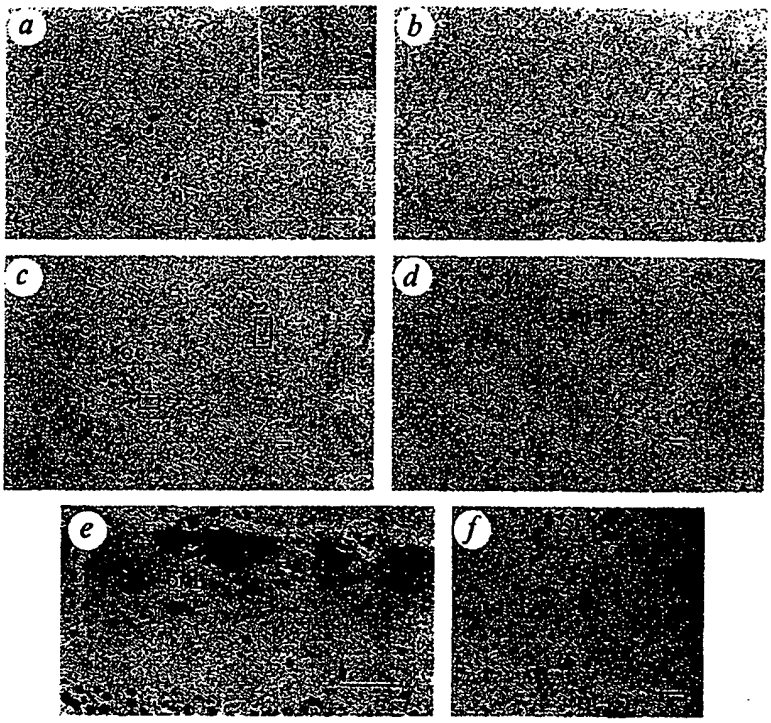

FIG. 2 Demonstration of AD-like amyloid plaques in PD-APP transgenic mice. Human and mouse brain sections were labelled with antiserum R1280 generated against synthetic human Aβ 1–40 peptide. H, Hippocampus; C, cortex; CC, corpus callosum; OML, outer molecular layer of the dentate gyrus; F, hippocampal fissure. Coronal sections of the hippocampus and neocortex from *a*, an 8-month-old transgenic mouse containing Aβ deposits (arrows), and *b*, a non-transgenic littermate. Inset in *a*, human tissue from AD frontal cortex stained with R1280. Adjacent parasaggital sections from a 13-month-old transgenic mouse before (*c*) and after (*d*) preincubation of the antibody with synthetic Aβ 1–40 peptide. In *c*, the large increase in Aβ was confined to the cortex and hippocampus. Several regions of the hippocampus contained densely packed Aβ, including the terminal zone of the perforant pathway in the outer molecular layer of the dentate gyrus (arrowheads). Boxed areas 1 and 2 are shown at higher magnification in *e* and *f*, respectively. Scale bars in *a–d*, 200 μm. In *e*, the outer molecular layer of the dentate gyrus contained areas of compacted and diffuse (asterisk) plaques. The edge of the granule cell layer is visible at the bottom. In *f*, a field of Aβ deposits in the occipital cortex. Scale bars in *e* and *f*, 40 μm.

METHODS. Mouse brains were removed and placed in Trojanowski's fixative[23] for 48 h before paraffin embedding. 6-μm coronal or parasaggital sections from transgenic and non-transgenic mice were placed adjacent to each other on poly-L-lysine coated slides. Sections were deparaffinized, rehydrated, and treated with 0.03% H_2O_2 for 30 min before overnight incubation at 4 °C with a 1:1,000 dilution of the Aβ antibody, R1280 (ref. 24). For absorption studies, synthetic human Aβ 1–40 peptide[25] in 10% aqueous dimethylsulphoxide was added to a final concentration of 7.0 μM to the diluted antibody and incubated for 2 h at 37 °C. The diluent was applied to the sections and processed under the same conditions as the standard antibody solution. Peroxidase rabbit IgG kit (Vector Labs) was then used as recommended, with 3,3'-diaminobenzidine (DAB) as the chromagen. Similarly fixed human AD brain was processed simultaneously under identical conditions.

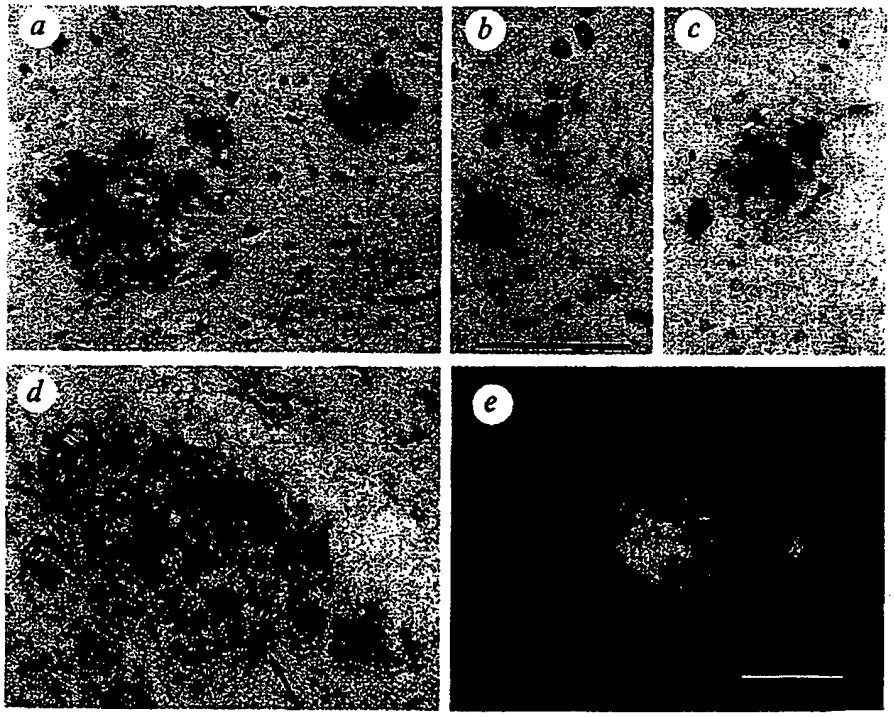

FIG. 3 Morphological diversity of Aβ deposition in the PD-APP mouse brain. Roughly spherical (*a*), and wispy, irregular deposits (*b*), labelled with antibody 9204 (Aβ1–5; ref. 26) specific for the free N terminus of Aβ. *c*, Aβ core and surround labelled with antibody 277-2, specific for the C terminus of Aβ1–42. *d*, Astrocytic gliosis (arrow) associated with Aβ deposition was evident after double immunolabelling with antibodies to glial fibrillary acidic protein (GFAP, red) and human Aβ (Aβ 1–5; brown). *e*, Aβ deposits were also reactive with thioflavin S. A compacted Aβ core and 'halo' is evident in the large plaque. The fine background fluorescence represents autofluorescent lipofuscin granules. Scale bars, 50 μm.

METHODS. For *a–c*, immunochemistry was performed as described for Fig. 2. Antibody 9204 (to Aβ1–5) was used at a concentration of 7.0 μg ml^{-1}. Antibody 277-2, specific for Aβ1–42 (apparent affinity for Aβ1–42 = 75 nM versus >10 μM for Aβ1–40 by radioimmunoassay competition), was prepared by immunizing New Zealand white rabbits with the peptide cysteine-aminoheptanoic acid–Aβ33–42 conjugated to cationized BSA ('Super Carriers'; Pierce) using a typical immunization protocol (500 μg per injection). Specific antibodies were affinity-purified from serum against the immunogen immobilized on agarose beads. Before incubation with 277-2 (10 μg ml^{-1}) sections were treated for 1–2 min with 80% formic acid. In *c*, the antibody 9204 was reacted using the peroxidase rabbit IgG kit (Vector Labs). The product was then visualized with DAB, and the sections were incubated overnight at 4 °C with a 1:500 dilution of polyclonal anti-GFAP (Sigma). The GFAP antibody was reacted using the alkaline phosphatase anti-rabbit IgG kit and alkaline phosphatase substrate kit I, (Vector Labs; used according to the manufacturer's recommendations). Sections were stained with thioflavin S using standard procedures[27] and viewed with ultraviolet light through an FITC filter of maximum wavelength 440 nm.

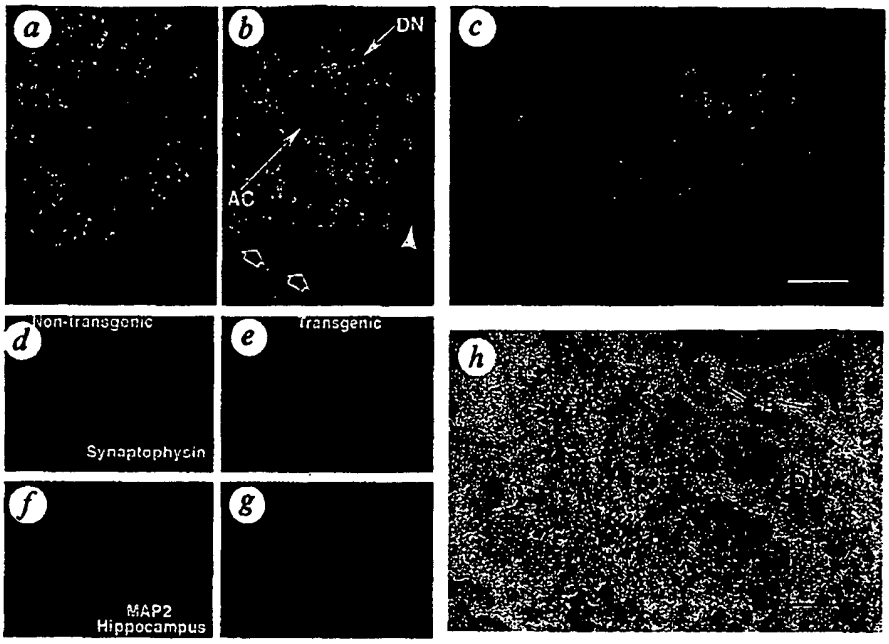

FIG. 4 Laser scanning confocal images of human and 8-month-old transgenic mouse brains, demonstrating the relationship of extracellular cortical Aβ deposit to dystrophic neurites and neuropil abnormalities, as well as the reduction of synaptic density and dendrites in the transgenic hippocampus. An Aβ deposit and adjacent neuropil in an 8-month-old transgenic mouse brain are also shown in an electron micrograph. In both human (*a*) and mouse (*b*) brains, Aβ deposits (red) were associated with distorted neurites (DN) containing synaptophysin (green). Yellow signifies overlap of the two markers. *b*, Aβ immunoreactive plaque in a transgenic mouse brain containing a central amyloid core (AC); synaptic loss (arrowhead) and compression (open arrows) of the neuropil surrounding the amyloid deposit are also evident. *c*, Aβ deposit (red) in a transgenic mouse brain associated with morphologically abnormal hAPP-positive neurites (green). The magnification for *a–g* is indicated by the scale bar in c (20 μm). Both synaptophysin (red) and microtubule-associated protein-2 (MAP-2; green) immunostaining were reduced throughout the molecular layer of the hippocampal dentate gyrus in the transgenic mouse (*e, g*) compared to the non-transgenic littermate (*d, f*). *h*, Immunoelectron micrograph of a transgenic mouse brain demonstrating extracellular Aβ deposition (A) decorated with the human-specific R1280 Aβ antiserum (outlined by arrows). A dystrophic neurite (DN) in the immediate vicinity of the Aβ deposit contained abundant large mitochondria (M) and laminar dense bodies (LB). Scale bar, 2 μm.

METHODS. 40-μm-thick vibratome sections were incubated overnight at 4 °C with the following antibodies: R1280 (1:1,000) in combination with polyclonal anti-synaptophysin in *a* and *b* (1:150; Dako) or 8E5 in *c* (7.0 μg ml^{-1}; described in Fig. 1). For *d–g*, sections were incubated with anti-synaptophysin or monoclonal anti-MAP 2 (1:20, Boehringer-Mannheim), and reacted with a goat anti-rabbit biotinylated antibody (1:100) followed by a mixture of FITC-conjugated horse anti-mouse IgG (1:75) and avidin D Texas red (1:100) (Vector Labs). The double-immunolabelled sections were viewed on a Zeiss Axiovert 35 microscope with attached laser confocal scanning system MRC 600 (Bio-Rad). The Texas red channel collected images of the R1280 (*a, b, c*) or synaptophysin (*d, e*) labelling, and the FITC channel collected synaptophysin (*a, b*), 8E5 (*c*), or MAP 2 (*f, g*) labelling. Optical *z*-sections 0.5 μm in thickness were collected from each region; details of similar image processing and storage are described in ref. 28. For immunoelectron microscopy, mice were perfused with saline followed by 2.0% paraformaldehyde and 1.0% glutaraldehyde in cacodylate buffer. 40-μm-thick vibratome sections were incubated with R1280, and reacted as in Fig 2. Immunolabelled sections with Aβ deposits were then fixed in 1.0% ammonium tetraoxide and embedded in epon/araldite before viewing ultrathin sections with a Jeol CX100 electron microscope[29].

also seen in another transgenic line generated from the PD-APP vector (line 35; data not shown). Aβ deposits of varying morphology clearly were evident as a result of using a variety of Aβ antibodies, including well characterized human-specific Aβ antibodies (Figs 2, 3) and antibodies specific for the free amino and carboxy termini of Aβ 1–42 (Fig. 3*a–c*). Serial sections demonstrated many plaques were positively stained with both of the latter antibodies. The forms of the Aβ deposition ranged from diffuse irregular types to compacted plaques with cores (Fig. 3). Non-transgenic littermates (Fig. 2*b*) showed none of these neuropathological changes. Immunostaining was fully absorbable with the relevant synthetic peptide (Fig. 2*d*), and was apparent using a variety of processing conditions, including fixation with paraformaldehyde and Trojanowski methods (Figs 2, 3). Many plaques were stained with thioflavin S (Fig. 3*e*), and some were also stained using the Bielschowsky silver method and were birefringent with Congo red (not shown), indicating the true amyloid nature of these deposits. Confirmation of the presence of extracellular Aβ was obtained using immunoelectron microscopy (Fig. 4*h*). The majority of plaques were intimately surrounded by GFAP-positive reactive astrocytes (Fig. 3*d*), similar to the gliosis found in AD plaques. The neocortices of the transgenic mice contained diffusely activated microglial cells, as defined by their amoeboid appearance and shortened processes (not shown). Preliminary attempts to identify neurofibrillary tangles with tau antibodies were negative, consistent with their well known absence in rodent tissues[14]. Nevertheless, clear evidence for neuritic pathology was apparent using both conventional and confocal immunomicroscopy. Many Aβ plaques were closely associated with distorted neurites that could be detected with hAPP-specific antibodies (Fig. 4*c*) and with anti-synaptophysin antibodies (Fig. 4*b*), suggesting that these neurites were derived in part from axonal sprouts, as observed in the AD brain (Fig 4*a*). The plaques compressed and distorted the surrounding neuropil (Fig. 4*b*), also as in the AD brain (Fig. 4*a*). Finally, synaptic and dendritic density were reduced in the molecular layer of the hippocampal dentate gyrus of the transgenic mice. This was evident by reduced immunostaining for the presynaptic marker synaptophysin (compare Fig. 4*d* and *e*) and the dendritic marker MAP-2 (compare Fig. 4*f* and *g*), as described in AD brain[15].

Several transgenic rodent lines have been produced that express either the hAPP gene or hAPP complementary DNAs regulated by a variety of promoters[4–10]. In particular, NSE-driven APP_{751} transgenic mice[9,10] have sparse Aβ deposits which are more typical of early AD and young Down's syndrome cases; in these mice, unlike ours, mature lesions such as frequent compacted plaques, neuritic dystrophy and extensive gliosis were very rare[10]. Our success in generating AD-like pathology consistently in these transgenic mice is probably due to the construct used and the high level of hAPP expression. The transgene contains a splicing cassette that permits expression of all three major hAPP isoforms. Expression is driven by the PDGF-β promoter, which is known to target expression preferentially to neurons

of the cortex, hippocampus, hypothalamus and cerebellum of transgenic animals[11]. The familial AD mutation at residue 717[12] may be important as it partially shifts production of Aβ from the 40-amino-acid form to the more amyloidogenic 42-residue peptide known to predominate in plaques[16,17]. Preparation of APP transgenic mice independently harbouring each of these features will be required to identify the essential component(s) that result in pathology.

The most notable feature of these transgenic mice is their Alzheimer-like neuropathology, which includes extracellular Aβ deposition, dystrophic neuritic components, gliosis and loss of synaptic density with regional specificity resembling that of AD. Based on the limited sampling to date, plaque density appears to increase with age in these transgenic mice, as it does in humans[1], implying a progressive Aβ deposition that exceeds its clearance, as also proposed for AD[18]. Our transgenic model provides strong new evidence for the primacy of APP expression and Aβ deposition in AD neuropathology and offers a means to test whether compounds that lower Aβ production and/or reduce its neurotoxicity *in vitro* can produce beneficial effects in an animal model prior to advancing such drugs into human trials. □

Received 17 October 1994; accepted 5 January 1995.

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ACKNOWLEDGEMENTS. We thank M. Mallory, A. Maya, N. Ge, E. Rockenstein, O. Stephenson and E. Johnstone for technical assistance; the animal care technicians involved in this project; J. Trojanowski for human brain sections; T. Saido and D. Selkoe for anti-Aβ antibodies 9204 and R1280, respectively; T. Collins and D. Goldgaber for the PDGF promoter and APP cDNA subclones, respectively; and D. Selkoe for helpful comments and advice.

Gating of the voltage-dependent chloride channel ClC-0 by the permeant anion

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CHLORIDE channels of the ClC family are important for the control of membrane excitability[1–3], cell volume regulation[4,5], and possibly transepithelial transport[6,7]. Although lacking the typical voltage-sensor found in cation channels[8–10], gating of ClC channels is clearly voltage-dependent. For the prototype *Torpedo* channel ClC-0 (refs 11–15) we now show that channel opening is strongly facilitated by external chloride. Other less permeable anions can substitute for chloride with less efficiency. ClC-0 conductance shows an anomalous mole fraction behaviour with Cl^-/NO_3^- mixtures, suggesting a multi-ion pore. Gating shows a similar anomalous behaviour, tightly linking permeation to gating. Eliminating a positive charge at the cytoplasmic end of domain D12 changes kinetics, concentration dependence and halide selectivity of gating, and alters pore properties such as ion selectivity, single-channel conductance and rectification. Taken together, our results strongly suggest that in these channels voltage-dependent gating is conferred by the permeating ion itself, acting as the gating charge.

The *Torpedo* electric organ Cl^--channel ClC-0 (ref. 12) has a 'slow' gate operating on both protochannels of the double-barrelled channel[13,14] simultaneously, and a 'fast' gate acting on single protochannels[13–15]. Both gates have opposite voltage-dependence, with the fast gate being opened by depolarization. ClC-0 was expressed in *Xenopus* oocytes and the fast gate was studied in isolation (Fig. 1*a*). The dependence of the steady-state open probability, p_{open}, on the transmembrane voltage V can be described by the Boltzmann distribution $p_{open} = 1/(1+\exp(z_n e_0(V_{1/2}-V)/kT))$ (where e_0 is the elementary charge, $V_{1/2}$ is the voltage of half-maximal activation, k is the Boltzmann constant, and T is the temperature) with a nominal gating charge $z_n \sim 1$ in agreement with earlier data[13,14]. Consistent with single-channel measurements[13,14], gating kinetics indicate a two-state gating mechanism. Reducing extracellular Cl^- concentration ($[Cl^-]_o$) shifts $p_{open}(V)$ to positive voltages without significantly changing its slope (the gating charge) (Fig. 1*b*). In contrast, intracellular chloride has little effect (Fig. 1*c*). A dependence of ClC-0 microscopic gating transitions on the Cl^--gradient has already been noted[15].

The dependence of gating on $[Cl^-]_o$ could be due to a simple mechanism in which channel opening depends on chloride binding to a site within the pore; p_{open} should then increase with $[Cl^-]_o$ and with positive intracellular potentials. Intracellular chloride has little effect on gating, because it cannot reach its binding site in the closed state.

The simplest model assumes that chloride-binding to a single site in the pore is required for channel opening[10]. Although it may serve as a first approximation to our data, the shift of p_{open} with $[Cl^-]_o$ is ~20% less than minimally predicted[10]. This suggests that this model is either principally unsuited to explain ClC-0 gating, or that it needs further refinement.

The pores of many channels, including certain chloride channels[16], can accommodate more than one ion, leading to concentration-dependent interactions within the pore. In a single-ion pore, conductance changes monotonously when the concentration ratio between two permeant ionic species is varied; in a multi-ion pore, however, interactions between different species can lead to a current minimum at a certain concentration ratio, an 'anomalous mole fracti n behaviour'[16,17]. We indeed found this effect with mixtures of Cl^- and NO_3^- (Fig. 2*b*), indicating that ClC-0 has a multi-ion pore. Thus, a model having a single chloride-binding site[10] may be too simplistic.

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ON THE COVER

A mouse model for Alzheimer's
Pages 523 and 476

Structure of a DNA repair enzyme
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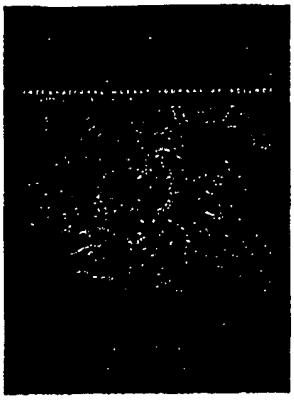
The trouble with gas giants
Pages 494 and 470

An isotopic puzzle
Page 496



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◀ The lack of an animal model has been hampered research into the pathogenesis of Alzheimer's disease. Previous efforts to transfer the human β-amyloid precursor protein (APP) into mice have failed to produce the characteristic amyloid plaque pathology, but the mice described on page 523 express high levels of a mutant APP, display amyloid plaques, and develop many other signs characteristic of Alzheimer's. Cover shows confocal image of plaque with reactive gliosis. See News and Views page 476.

THIS WEEK...

DNA repair kit

Uracil–DNA glycosylases are responsible for initiating repair of uracil residues in DNA, one of the commonest forms of mutagenic lesion, and the report of the three-dimensional structure of such an enzyme throws light on the structural basis of DNA damage repair. The structure of herpes simplex virus uracil–DNA glycosylase alone, and in complexes with uracil base and with an oligonucleotide, suggests binding models for both single- and double-stranded substrates. Page 487.

Gas giants an endangered species?

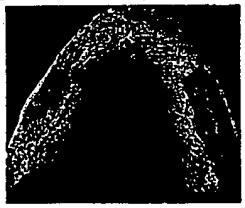
Are 'gas-giant' planets such as Jupiter and Saturn rarities? Only relatively young stars are surrounded by the large quantities of gas that are required to form gas giants, so by the 10^7 years or so required for planet formation according to current models, much of the gas will have escaped. Direct observations of molecular gas around stars in the critical age range of 10^6–10^7 years now show that after only a few million years, the gas remaining is insufficient to make a Jupiter-size planet. Planet formation must accordingly be much quicker than present models suggest. Pages 494 and 470.

Isotopic puzzle from space

Primitive meteorites are isotopically heterogeneous, reflecting the composition of material from the Solar System. Planetary or asteroidal material, however, has been thermally 'processed' and is expected to be isotopically homogeneous. The Acapulco meteorite is a fragment of an asteroidal body that has experienced heating and partial melting, yet, as El Goresy *et al.* report, it includes graphite grains retaining the isotopic signatures of a diverse range of precursor materials. Page 496.

Caucasus race

The discovery in Dmanisi, East Georgia, of the well preserved fossil jawbone of an early human has aroused great interest because the region is a possible 'gateway to Europe', where *Homo erectus* was suspected but not documented. A fossil mandible (right), found in 1991, is now described in detail (pages 509 and 472); it is the earliest example of *H. erectus* to be found in western Eurasia (1.6–1.8 million years old) and shows similarities to African and Chinese members f the species.



OPINION

NEWS

CORRESPONDENCE

NEWS AND VIEWS

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Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 1550–1555, February 1997
Neurobiology

Amyloid precursor protein processing and $A\beta_{42}$ deposition in a transgenic mouse model of Alzheimer disease

(PDAPP mouse/β-peptide/amyloidogenesis)

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Communicated by L. Iversen, University of Oxford, Oxford, United Kingdom, December 6, 1996 (received for review August 18, 1996)

ABSTRACT **The PDAPP transgenic mouse, which overexpresses human amyloid precursor protein (APP717V→F), has been shown to develop much of the pathology associated with Alzheimer disease. In this report, levels of APP and its amyloidogenic metabolites were measured in brain regions of transgenic mice between 4 and 18 months of age. While absolute levels of APP expression likely contribute to the rate of amyloid β-peptide (Aβ) deposition, regionally specific factors also seem important, as homozygotic mice express APP levels in pathologically unaffected regions in excess of that measured in certain amyloid plaque-prone regions of heterozygotic mice. Regional levels of APP and APP-β were nearly constant at all ages, while Aβ levels dramatically and predictably increased in brain regions undergoing histochemically confirmed amyloidosis, most notably in the cortex and hippocampus. In hippocampus, Aβ concentrations increase 17-fold between the ages of 4 and 8 months, and by 18 months of age are over 500-fold that at 4 months, reaching an average level in excess of 20 nmol of Aβ per g of tissue. $A\beta_{1-42}$ constitutes the vast majority of the depositing Aβ species. The similarities observed between the PDAPP mouse and human Alzheimer disease with regard to $A\beta_{42}$ deposition occurring in a temporally and regionally specific fashion further validate the use of the model in understanding processes related to the disease.**

In the Alzheimer disease (AD) brain, region-specific amyloid β-peptide (Aβ) amyloidosis is a key pathological feature and is accompanied by astrogliosis, microgliosis, cytoskeletal changes, and synaptic loss. These pathological alterations are thought to be linked to the cognitive decline that clinically defines the disease (1). AD primarily afflicts the elderly, although genetic mutations in the amyloid precursor protein (APP) gene have been described that accelerate the disease process and lower the average age of onset by decades, further supporting a fundamental role for this protein in the disease (2–5). Many questions remain about the spatial–temporal sequence of neuropathological events, particularly what factors are responsible for the selective vulnerability of certain brain regions to amyloidosis. Candidate mechanisms include constitutive increased production of Aβ in vulnerable areas, age-related changes in expression of APP and production of Aβ, and inherent differences in the ability of different brain regions to clear or catabolize Aβ. These fundamental issues are not easily addressed in human subjects.

Similar neuropathology to that seen in human AD brain has been demonstrated in a transgenic mouse generated using a platelet-derived growth factor β promoter driving a human APP minigene (6) and possessing the familial AD mutation V→F at APP position 717 (4) (PDAPP). These animals express high levels of APP and Aβ, but more importantly they exhibit profuse Aβ amyloidosis, which, in an age- and brain region-specific manner, morphologically resembles that seen in AD. In addition, these mice develop marked astrogliosis, microgliosis, cytoskeletal changes, and synaptic loss. They offer the opportunity to examine the biological events leading to amyloidosis and synaptic loss and provide an effective animal model to test for therapeutic agents that have the ability to retard or interfere in these pathological processes.

In this report, we quantitatively assess the profile of a number of APP-derived protein species in different brain regions at various ages in these PDAPP transgenic mice. This is addressed through the use of enzyme-linked immunoassays (ELISAs) configured with antibodies specific to Aβ, $A\beta_{1-42}$, APP cleaved at the β-secretase site (7), and APP containing the first 12 aa of Aβ [i.e., α-secretase-cleaved (8) and full-length (FL) APP]. These biochemical measurements were then compared with the regional distribution of amyloid plaques visualized immunohistochemically. The results suggest that age, Aβ production levels, and brain region-specific factors all likely play critical roles in amyloid deposition in the PDAPP mouse. Striking similarities in both the regional distribution and depositing form of Aβ are noted between the mouse model and the human AD condition. Because of the magnitude and temporal predictability of Aβ deposition, the PDAPP mouse is a practical model in which to test agents that either inhibit the processing of APP to Aβ or retard Aβ amyloidosis.

MATERIALS AND METHODS

Transgenic Animals. The founder of PDAPP line 109 was produced on a Swiss Webster × $B_6D_2F_1$ (C57Bl/6 × DBA/2) background (all strains from Taconic Farms) and bred for three generations with animals of the same background. Generation 3 was bred with $B_6D_2F_1$. Generation 4 was bred with Swiss Webster to produce the outbred heterozygous animals used for these experiments, except where noted. Generation 4 heterozygous animals were bred together to obtain a homozygous animal colony. Generation 4 animals were also bred with C57Bl/6 (The Jackson Laboratory) for five generations to produce a line with a more inbred background. Gross effects on longevity have not been observed in the transgenic lines compared with littermate controls.

Brain Tissue Preparation. The heterozygote transgenic (6, 9) and nontransgenic littermate animals were perfused intracardially with ice-cold 0.9% saline. The brain was removed and one hemisphere was prepared for immunohistochemical anal-

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0027-8424/97/941550-6$2.00/0
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Abbreviations: AD, Alzheimer disease; Aβ, amyloid β-peptide; FL, full-length; RT, room temperature.

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ysis, while three brain regions (cerebellum, hippocampus, and cortex) were dissected from the other hemisphere and used for Aβ and APP measurements. For comparative studies of homozygous and heterozygous animals, an additional sample enriched in thalamic matter was dissected.

Tissue for ELISAs was homogenized in 10 volumes of ice-cold guanidine buffer (5.0 M guanidine·HCl/50 mM Tris·Cl, pH 8.0). The homogenates were mixed for 3 to 4 hr at room temperature (RT), then either assayed or stored at −20°C before quantitation of Aβ and APP. Preliminary experiments showed the analytes were stable to this storage condition and that synthetic Aβ peptide (Bachem) could be quantitatively recovered when spiked into littermate control brain tissue homogenates (data not shown).

Aβ Measurements. The brain homogenates were further diluted 1:10 with ice-cold casein buffer (0.25% casein/0.05% sodium azide/20 μg/ml aprotinin/5 mM EDTA, pH 8.0/10 μg/ml leupeptin in PBS) before centrifugation (16,000 × *g* for 20 min at 4°C). The Aβ standards (1–40 or 1–42 aa) were prepared such that the final composition included 0.5 M guanidine in the presence of 0.1% bovine serum albumin (BSA).

The "total" Aβ sandwich ELISA consists of the capture antibody 266, which is specific to amino acids 13–28 of Aβ (10), and the biotinylated reporter antibody 3D6, which is specific to amino acids 1–5 of Aβ. The 3D6 antibody does not recognize secreted APP or APP-FL but detects only Aβ species with amino-terminal aspartic acid. The assay has a lower limit of sensitivity of ≈50 pg/ml (11 pM) and showed no crossreactivity to the endogenous murine Aβ peptide at concentrations up to 1 ng/ml (data not shown).

The configuration of the $A\beta_{1-42}$-specific sandwich ELISA employs the capture antibody mAb 21F12 ($A\beta_{33-42}$). Biotinylated 3D6 is also the reporter antibody in this assay, which has a lower limit of sensitivity of ≈125 pg/ml (28 pM; data not shown). An $A\beta_{x-42}$ sandwich ELISA, using 266 as the capture antibody and biotinylated 21F12 as the reporter antibody, was used on a subset of brain homogenates. The low end sensitivity of this assay is ≈250 pg/ml (56 pM; data not shown).

The 266 and 21F12 mAbs were coated at 10 μg/ml into 96-well immunoassay plates (Costar) overnight at RT. The plates were then aspirated and blocked with 0.25% human serum albumin in PBS buffer for at least 1 hr at RT, then stored dessicated at 4°C until use. The plates were rehydrated with wash buffer (0.05% Tween 20 in tris-buffered saline) before use. The samples and standards were added to the plates and incubated at RT for 1 hr. The plates were washed three or more times with wash buffer between each step of the assay.

The biotinylated 3D6, diluted to 0.5 μg/ml in casein assay buffer (0.25% casein/0.05% Tween 20, pH 7.4, in PBS), was incubated in the wells for 1 hr at RT. Avidin–horseradish peroxidase (Vector Laboratories), diluted 1:4000 in casein assay buffer, was added to the wells for 1 hr at RT. The colorimetric substrate, Slow TMB-ELISA (Pierce), was added and allowed to react for 15 min, after which the enzymatic reaction was stopped with addition of 1 M H_2SO_4. Reaction product was quantified using a Molecular Devices Vmax spectrophotometer measuring the difference in absorbance at 450 nm and 650 nm.

APP ELISAs. Two different APP assays were used (see Fig. 1). The first recognizes APP-α and APP-FL, while the second recognizes APP-β [APP ending at the methionine preceding the Aβ domain (7)]. The capture antibody for both the APP-α/FL and APP-β assays is 8E5 (6). The reporter mAb (2H3) for the APP-α/FL assay was generated against amino acids 1–12 of Aβ. The lower limit of sensitivity for the 8E5/2H3 assay is ≈11 ng/ml (150 pM). For the APP-β assay, the polyclonal antibody 192, specific to the carboxyl terminus of the β-secretase cleavage site of APP (7), was used as the reporter. The lower limit of sensitivity of the 8E5/192 assay is ≈43 ng/ml (600 pM).

For both APP assays, the 8E5 mAb was coated onto 96-well Costar plates as described above for 266. Purified recombinant secreted APP-α (the secreted form of APP 751) and APP596 of the 695 form were the reference standards used for the APP-α/FL and APP-β assays, respectively (8). The 5 M guanidine brain homogenate samples were diluted 1:10 in specimen diluent for a final buffer composition of 0.5 M NaCl, 0.1% Nonidet P-40, and 0.5 M guanidine. The APP standards and samples were added to the plate and incubated for 1.5 hr at RT. Biotinylated reporter antibodies 2H3 and 192 were incubated with samples for 1 hr at RT. Streptavidin–alkaline phosphatase (Boehringer Mannheim), diluted 1:1000 in specimen diluent, was incubated in the wells for 1 hr at RT. The fluorescent substrate 4-methyl-umbellipheryl-phosphate, was added, and the plates were read on a Cytofluor 2350 (Millipore) at 365 nm excitation and 450 nm emission.

mAb Production. The immunogens for 3D6 ($A\beta_{1-5}$), 2H3 ($A\beta_{1-12}$), 2G3 ($A\beta_{33-40}$), 21F12 ($A\beta_{33-42}$), and 12H7 ($A\beta_{33-42}$) were separately conjugated to sheep anti-mouse immunoglobulin (Jackson ImmunoResearch). Mice were immunized and hybridomas were generated by standard methods. The hybridoma supernatants were screened for high-affinity mAbs by RIA as previously described (10).

Antibodies 12H7 and 21F12 were demonstrated to show negligible crossreactivity (<0.4%) with $A\beta_{1-40}$ in either ELISA or competitive RIA. Antibody 2G3 was similarly shown to be nonreactive with $A\beta_{1-42}$.

Immunohistochemistry. The tissue from one brain hemisphere of each mouse was drop-fixed in 4% paraformaldehyde and postfixed for 3 days. The tissue was mounted coronally and 40-μm sections were collected using a vibratome. The sections were stored in antifreeze solution (30% glycerol/30% ethylene glycol in 40 mM NaPO4) at −20°C before immunostaining. Every sixth section, from the posterior cortex through the hippocampus, was incubated with the appropriate biotinylated antibody (either 3D6, 2G3, or 12H7) at 4°C, overnight. The sections were then reacted with the horseradish peroxidase–avidin–biotin complex (Vector Laboratories) and developed using 3,3′-diaminobenzidine (DAB) as the chromagen.

RESULTS

Aβ and APP Assays. Fig. 1 illustrates the recognition sites of antibodies used in the Aβ and APP assays. The APP-α/FL assay recognizes secreted APP including the first 12 aa of Aβ. Since the reporter antibody (2H3) is not specific to the α-clip site, occurring between Aβ amino acids 16 and 17 (8), this assay also recognizes APP-FL. Preliminary experiments using immobilized APP antibodies to the cytoplasmic tail of APP-FL to deplete brain homogenates of APP-FL suggest that ≈30–40% of the APP-α/FL APP is APP-FL (data not shown). Due to the specificity of the polyclonal reporter antibody, the APP-β assay recognizes only the APP clipped immediately amino-terminal to Aβ (7).

Aβ immunoreactivity was characterized by size exclusion chromatography (Superose 12, Pharmacia) of brain homogenates. Comparisons were made of 2-, 4-, and 12-month-old transgenic brain specimens as well as a 12-month-old nontransgenic mouse brain homogenate to which $A\beta_{1-40}$ had been spiked at a level roughly equal to that found in the 12-month-old transgenic mice. The elution profiles of the transgenic brain homogenates were similar in that the peak fractions of Aβ immunoreactivity occurred in the same position, a single broad symmetric peak that was coincident with the immunoreactive peak of spiked $A\beta_{1-40}$. Attempts were then made to immunodeplete the Aβ immunoreactivity using resin-bound antibodies against Aβ (mAb 266 against $A\beta_{13-28}$), the secreted forms of APP (mAb 8E5 against $APP_{444-592}$ of the 695 form),

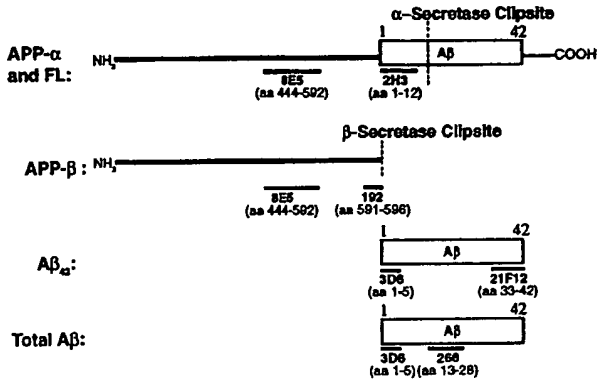

FIG. 1. Immunoassays to forms of Aβ and APP described in the text. Immunoassays were prepared to measure APP-α/FL, APP-β (secreted APP ending at the methionine preceding the start of the Aβ region), and total Aβ. Antibodies are mouse mAbs except 192, which is an affinity-purified rabbit polyclonal antibody. Antibodies 192 and 21F12 are specific to fragments of APP and Aβ, respectively, with carboxyl termini as indicated. Antibody 3D6 is specific for $Aβ_{1-5}$ and does not cross react with APP-α/FL. Methodologies used for the immunoassays are described in text.

the carboxyl terminus of APP (mAb 13G8 $APP_{676-695}$ of the 695 form), or heparin agarose. Only the 266 resin captured Aβ immunoreactivity (data not shown), demonstrating that APP-FL or carboxyl-terminal fragments of APP are not contributing to the Aβ measurement. The $Aβ_{42}$ ELISA uses a capture antibody that recognizes $Aβ_{42}$ but not $Aβ_{1-40}$ peptide. The $Aβ_{42}$ assay, like the total Aβ assay, is not affected by the FL or carboxyl-terminal forms of APP containing Aβ in the homogenates as shown by similar immunodepletion studies (data not shown).

Total Aβ and APP Measurements. Fig. 2 shows the levels of total Aβ, APP-α/FL, and APP-β in the hippocampus, cortex, and cerebellum of transgenic mice as a function of age. Each data point represents the mean value for each age group. The relative levels of APP-α/FL and APP-β in all three brain regions remain relatively constant over time. The hippocampus expresses the highest levels of both APP-α/FL and APP-β followed by the cortex and cerebellum, respectively. The mean ± SD values of all ages for APP-α/FL and APP-β levels in the hippocampus are 720 ± 135 pmol/g and 171 ± 17 pmol/g, respectively. In the hippocampus, the levels of APP-α/FL are approximately 3.5- to 6.0-fold higher than that of APP-β at all ages. Since APP-α/FL is 30–40% APP-FL (see above), we estimate the pool of brain APP to consist of ≈50% APP-α, 30% APP-FL, and 20% APP-β.

Since there was a 1.6-fold increase of APP-α/FL in the hippocampus, which displays robust pathology, versus that of the comparatively unaffected cerebellum, we wanted to determine whether this modest increase of transgenic APP expression was the determinant of the regional pathology displayed in this transgenic line. Western blot analysis of APP transgene expression was performed on brain regions from either heterozygous or homozygous transgenic mice (Fig. 3), both of which show the same regional distribution of pathology (data not shown). There are higher levels of APP expression in the thalamus of the homozygous animal than in the hippocampus of the heterozygous animal; yet pathology in the hippocampus of heterozygotes is extensive with early onset, and the thalamus only displays a minor amount of pathology at later ages. Likewise, there are higher levels of transgene expression in the cerebellum of the homozygous animal, a largely unaffected region, than in the cortex of the heterozygous animal, a region with robust pathology. The same type of comparative analysis was performed on Aβ levels, determined by ELISA, in various brain regions of 2-month-old heterozygous and homozygous transgenic animals (Fig. 3). Although higher levels of Aβ are present in the susceptible brain regions than in unaffected regions in the heterozygotes, the Aβ levels in the thalamus of the homozygotes, which show only minimal pathology in older animals, is equivalent to that in the cortex of the heterozygotes, a region displaying robust pathology at an early age.

In contrast to APP levels, Aβ levels increase dramatically with age in the hippocampus and cortex, with the greatest increase in the hippocampus. No such increase was noted in the cerebellum of the PDAPP transgenic mice (Fig. 2). These region-specific increases of Aβ correlate with the 3D6 immunohistochemical results (Fig. 4 and below). Compared with the levels of 4-month-old mice, Aβ levels increase 8-fold by 8 months of age and 400-fold at 18 months of age in cortex (6330 ± 2310 pmol of Aβ per g of tissue at age 18 months). The corresponding increases in Aβ observed in hippocampus are even more impressive, as the 8-month value is 17 times that at 4 months and increases to 500-fold at 18 months of age (20,800 ± 5250 pmol of Aβ per g of tissue at 18 months).

$Aβ_{42}$ Measurements in Transgenic Mouse Brain. We next determined if, as in human AD subjects (11, 12), the depositing Aβ is the longer $Aβ_{1-42}$ form by measuring the levels of $Aβ_{1-42}$ in the hippocampus and cortex of transgenic mice at different ages. As shown in Table 1, the increase in Aβ observed with age in the hippocampus and cortex of transgenic mice is due

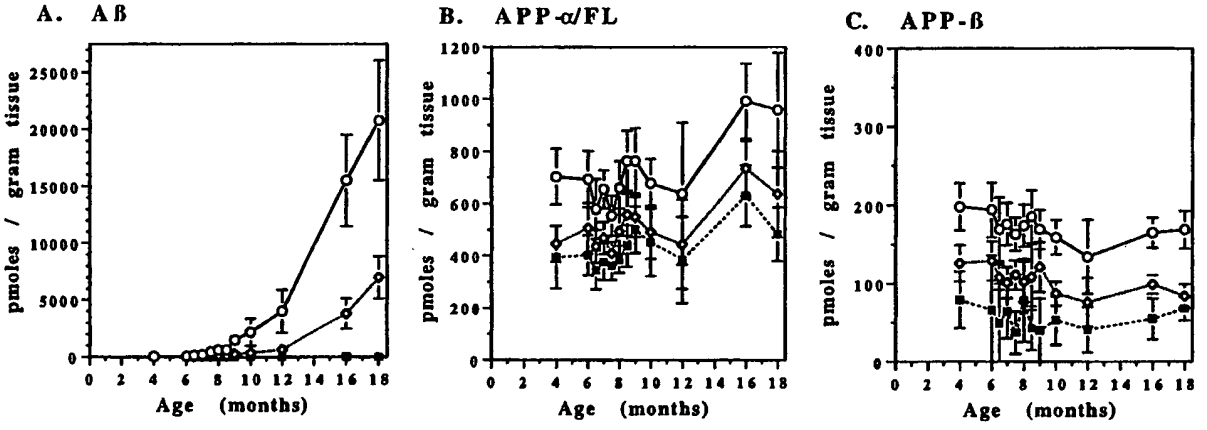

FIG. 2. Age-dependent changes in brain Aβ and APP levels in the PDAPP transgenic mice. PDAPP mice were sacrificed at the ages indicated and levels of Aβ (*A*), APP-β (*B*), and APP-α/FL (*C*) were determined in the cortex (◇), hippocampus (○), and cerebellum (■) by ELISA. Values represent the means ± SD of 9–14 animals.

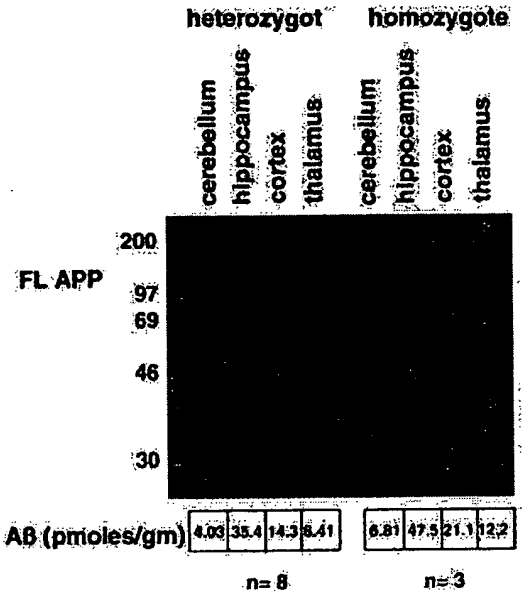

FIG. 3. Levels of APP and Aβ in brain regions of heterozygous and homozygous PDAPP transgenic mice. Two-month-old PDAPP mice were sacrificed, and the amount of APP-FL was measured by Western blot analysis using an APP carboxyl-terminal antibody (anti-6; ref. 6) in hippocampus, cortex, cerebellum, and thalamus of heterozygous and homozygous mice. Essentially similar results were obtained from Western blot analyses using other APP antibodies (human-specific 8E5 and 2H3; data not shown). The amount of Aβ in these same regions from eight heterozygous and three homozygous 2-month-old animals was determined by ELISA, and the average levels are listed.

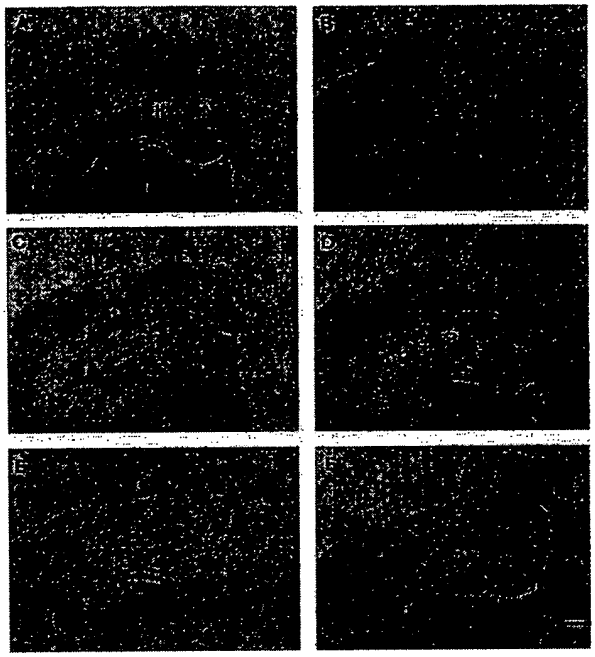

FIG. 4. Age-dependent increases in Aβ plaque burden in the PDAPP mouse. Aβ deposits in the opposite hemisphere of brains used for Aβ and APP ELISAs at 4 (*A*, arrow indicates deposit), 8 (*B*), 10 (*C*), 12 (*D*), 16 (*E*), and 18 (*F*) months of age. Brains are shown from mice with Aβ ELISA values that correspond to the mean of their age group. Deposition typically occurs in an age- and region-dependent manner, with early and heavy involvement of the frontal cortex (F) and hippocampus (H), while the underlying thalamus (T) is devoid of plaques. Arrows in *D* outline the outer molecular layer of the dentate gyrus, which contains terminals from the perforant pathway. [Bar (in *F*) = 500 μm.]

to $A\beta_{1-42}$. $A\beta_{1-42}$ constituted 27% of the 17 pmol/g of the Aβ present in the brains of young animals, this percentage increased to 89% of the 694 pmol/g in 12-month-old animals. Since the $A\beta_{1-42}$-specific assay does not detect $A\beta_{42}$ with a truncated or modified amino terminus, further analysis of the $A\beta_{42}$ species in the transgenic mice was performed. A subset (n = 4) of 12-month-old PDAPP transgenic mice cortical homogenates were quantitated in the sandwich ELISA measuring $A\beta_{x-42}$ as well as the $A\beta_{1-42}$-specific assay. Approximately 90% of the $A\beta_{42}$ is true $A\beta_{1-42}$ and the remaining 10% begins somewhere other than the amino-terminal aspartic acid of Aβ.

Aβ Measurements in Outbred and Inbred Strains of PDAPP Transgenic Mice. The levels of total Aβ in the hippocampus and cortex were compared between the outbred (n = 14) and inbred strains (n = 20) of PDAPP mice at 4 months of age and found to be 15.95 ± 2.70 and 15.51 ± 1.72 pmol/g of tissue in the cortical homogenates and 38.08 ± 6.76 and 33.02 ± 4.56 pmol/g of tissue in the hippocampal homogenates, respectively. Statistical analysis of the cortical and hippocampal Aβ measurements determined that there was not a significant difference in the interanimal variability between the two groups (data not shown).

Aβ Immunohistochemistry in PDAPP Transgenic Brain. To correlate Aβ accumulation in brain as measured by ELISA with the deposition of Aβ into plaques as measured immunohistochemically, opposite hemispheres were sectioned and immunoreacted with 3D6. Fig. 4 illustrates the progression of Aβ deposition in 4-, 8-, 10-, 12-, 16-, and 18-month-old animals, with Aβ measurements representative of the mean value of their age group. At 4 months of age, transgenic brains contain small, rare, punctate deposits, ≈20 μm in diameter, that were only infrequently observed in the hippocampus as well as the frontal and cingulate cortex. By 8 months of age, these regions contain a number of thioflavin-positive Aβ aggregates (data not shown) that form plaques as large as 150 μm in diameter. At 10 months of age, many large Aβ deposits are found throughout the frontal and cingulate cortex and the molecular layers of the hippocampus. The outer molecular layer of the dentate gyrus receiving perforant pathway afferents from the entorhinal cortex is clearly heavily delineated by Aβ deposition. This general pattern was more pronounced by heavier Aβ deposition at 1 year of age, and by 18 months of age it involves most of the neocortex. Notably, a striking increase in Aβ plaque burden paralleled the rising Aβ levels (compare Figs. 2*A* and 4). Staining of sections with antibodies specific for $A\beta_{42}$ (Fig. 5*A*) and $A\beta_{40}$ (Fig. 5*B*) indicates that amyloid plaques are primarily composed of $A\beta_{42}$, again paralleling the ELISA results. These findings strongly argue that the rise in brain $A\beta_{42}$ concentration determined by ELISA is due to the age-dependent amyloidosis.

Table 1. Aß levels in the cortex of transgenic brain (pmol of Aß per g of wet tissue)

Age, months	n	$A\beta_{1-42}$	Total Aß	% $A\beta_{42}$
4	11	4.7 ± 1.3	17 ± 3.4	27
8	13	76 ± 40	112 ± 64	68
10	5	247 ± 133	248 ± 139	99
12	9	615 ± 333	694 ± 403	89
16	10	3538 ± 1104	3813 ± 1327	93
18	10	5612 ± 1583	6332 ± 2310	89

Values are in mean ± SD.

DISCUSSION

Aβ amyloidosis is an established diagnostic criteria of AD (13, 14) and is consistently seen in higher-order cortical areas as well as the hippocampal formation of the brain in affected subjects. It is believed that Aβ amyloidosis is a relatively early event in the pathogenesis of AD that subsequently leads to neuronal dysfunction and dementia through a complex cascade of events (15, 16). For unknown reasons, other brain regions, such as the cerebellum, are typically spared from advanced forms of amyloidosis in AD. Both the sequence of events of this process as well as the brain region specificity of AD pathology have been extraordinarily difficult to unravel because brain tissue cannot typically be analyzed until after the death of these patients. Recently, reliable and robust Aβ amyloidosis accompanied by neuropathology has been demonstrated in the PDAPP mouse (6), providing a model in which to study these issues. In this report we have investigated the key metabolites of APP as a function of age and anatomical location and compared this to the immunohistochemically detected changes of Aβ in these animals.

Various pathways of APP processing have been described, including the α-secretase pathway in which cleavage of APP occurs within Aβ (Fig. 1 and ref. 8) and the amyloidogenic or β-secretase pathway in which cleavage of APP occurs at the amino terminus of Aβ (Fig. 1 and ref. 7). Further cleavage of APP leads to the constitutive production of Aβ, including the form ending at position 42 ($A\beta_{42}$). We have taken advantage of site-specific antibodies to develop ELISAs that detect specific APP products arising from these individual pathways in the PDAPP mouse brain.

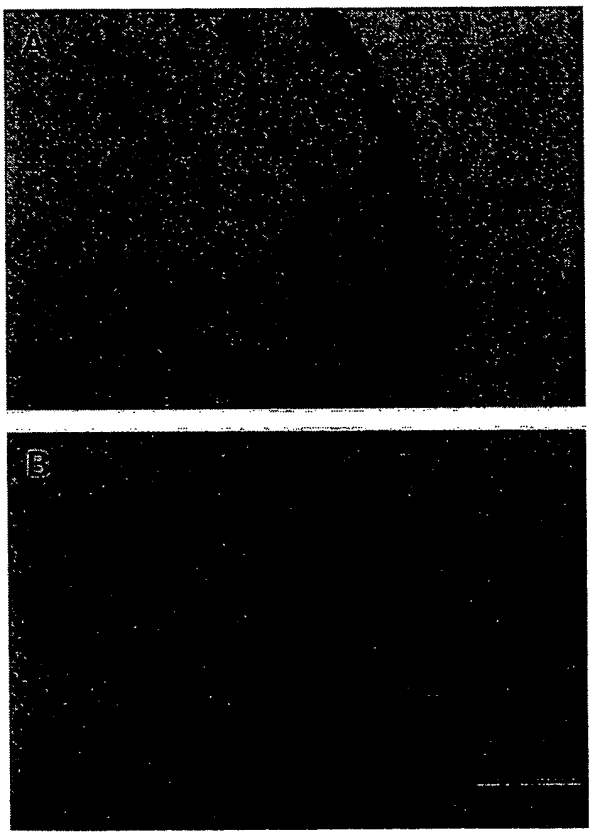

FIG. 5. $A\beta_{42}$ and $A\beta_{40}$ immunohistochemical analysis in 18-month-old PDAPP mice. Aβ deposits in adjacent sections from an 18-month-old mouse visualized with antibodies specific for $A\beta_{42}$ (*A*) and $A\beta_{40}$ (*B*). [Bar (in *B*) = 100 μm.]

Analysis of Aβ and APP immunoreactivities in the PDAPP mouse brain leads to several interesting conclusions. First, levels of APP-α/FL were relatively constant over the age of the PDAPP mice examined and varied only modestly (1.6-fold) among the brain regions analyzed (Fig. 2), indicating that age- or region-dependent changes in expression of the transgene are not amyloidogenic factors in this animal. The lack of amyloid deposition and pathology in the unaffected brain regions in the presence of high levels of APP expression strongly argues that APP overexpression alone is insufficient to cause amyloid deposition in this model. An additional finding that supports this observation is that mice homozygotic for the PDAPP minigene have thalamic levels of APP that exceed those seen in the hippocampus of the heterozygote animals and yet still do not display Aβ deposition in this region (Fig. 3).

To test if region- or age-dependent differential processing of APP to Aβ contributes to Aβ deposition in the PDAPP mouse, we measured the β-secretase product of APP-FL (APP-β) at various ages in different brain regions (7). APP-β is a direct product of β-secretase activity, and its production parallels the production of Aβ *in vitro* under conditions that are expected to either directly modulate the activity of β-secretase or to modulate the accessibility of APP to β-secretase (17–19). Levels of APP-β are therefore thought to correlate with the production of Aβ. The PDAPP mouse thus affords the unique opportunity to measure levels of this metabolite in a tissue destined to undergo amyloidosis at different stages of deposition. Examination of APP-β levels in different brain regions of the PDAPP mouse shows that levels of this APP metabolite do not change significantly with age (Fig. 2). This is true even in the hippocampus where very significant Aβ levels and deposition occur at 8 months of age or greater (Figs. 2 and 4). This finding argues strongly that the age-related amyloid deposition seen in the PDAPP mice is not due to age-dependent increased processing of APP to Aβ mediated by the β-secretory pathway.

Vulnerable brain regions do seem to intrinsically process more APP to Aβ, however. The initial Aβ levels in brains of young animals, before amyloidotic deposition, are higher in hippocampus and cortex than in cerebellum (38.1 pmol/g Aβ in hippocampus, 4.1 pmol/g Aβ in the cerebellum). Levels of APP-β are also higher in amyloid-depositing brain regions; they are 3-fold higher in hippocampus and 2-fold higher in cortex relative to the unaffected cerebellum (Fig. 2). Even normalizing for the 1.5-fold difference in transgenic APP expression between these brain regions, there is 2-fold more APP-β and 7-fold more Aβ in the hippocampus compared with cerebellum, supporting the notion that there is more efficient processing of APP to Aβ in affected brain regions than in unaffected regions. Modest changes in Aβ production, such as in Down syndrome, are sufficient to accelerate amyloid deposition (15). Taken together, these data suggest that increased constitutive processing to Aβ, via the β-secretase pathway, is a significant factor in the brain region specific deposition of Aβ that is seen in the PDAPP mouse.

However, there must be other significant factors in addition to enhanced Aβ production that lead to amyloidosis, since measurements of Aβ or APP-β levels in unaffected brain regions of mice homozygotic for the PDAPP minigene are essentially equivalent to those seen in affected brain regions of the heterozygote PDAPP mice (Fig. 3). This clearly indicates that not only is reaching a threshold level of Aβ required to cause amyloid deposition, but that other regional specific factors are required to interact with Aβ to elicit amyloid deposition. One can only speculate that such factors might include age-dependent expression of specific proteoglycans (20) or specific receptors and binding substances for Aβ such as C1q, Apo E, or APO J (21–23). Such factors may interact with the Aβ peptide and result in its increased aggregation or

fibril formation. The defined regional and time course of amyloid deposition events in this model allow a means to define these factors.

Initial brain levels of Aβ show interanimal variability up to 2-fold. There are no outlying animals with excessively high or absent Aβ. Since the genetic strain of mice can have an effect on transgene phenotype and this line was derived in a highly outbred background, we tested whether the variability could be reduced by crossing it onto an inbred strain. Variability among animals of initial brain Aβ levels does not seem to be due to the genetic variability of the outbred strain since inbred animals displayed the same variability in Aβ as the outbred animals.

Aβ amyloid deposition seen in the PDAPP mouse brain is highly age- and region-specific (Figs. 2 and 4). Amyloid deposition accelerates at around 7 months of age, and by 12 months of age, amyloid deposition is pronounced throughout the hippocampus and in the frontal region of the cortex. Between 12 and 16 months of age, a further dramatic increase in deposition is observed. This anatomical localization of Aβ deposition is remarkably similar to that seen in AD (13). The age-dependent increases in immunohistochemically detectable amyloid deposition correlate well with the dramatic rise in Aβ levels in these brain regions as measured by ELISA. An increase in Aβ is measurable by 7 months of age and by 10 months the hippocampus has 2180 pmol Aβ/g of tissue. By 18 months of age, the levels of $A\beta_{42}$ are comparable to the higher Aβ levels observed in humans with AD (24). Aβ levels in the cerebellum at 10 months, an unaffected brain region, remain at 4 pmol/g of tissue—essentially unchanged relative to the levels at 4 months of age, in agreement with the extent of amyloid deposition observed by histological analyses. Thus, a reproducible increase in measurable Aβ occurs in the brain tissue of the PDAPP mice that correlates with the severity of amyloid deposition. These results suggest that in aged PDAPP mice, monitoring of brain Aβ levels reflects amyloid burden and therefore direct immunoassay measurement of brain Aβ levels can be used to monitor the effects of compounds that reduce amyloid plaque burden.

The vast majority of depositing Aβ in these mice is of the longer $A\beta_{42}$ form, despite the fact that the majority of Aβ produced in younger animals are shorter species (Table 1). The ELISA data suggest that $A\beta_{42}$ is preferentially depositing in the transgenic mice, a result confirmed by immunostaining (Fig. 5). This is in agreement with studies of human AD and of Down syndrome brains, wherein the predominant and initially depositing Aβ is the longer form (11). In this respect, this mouse model again faithfully reproduces human AD pathology, indicating that the biological mechanisms leading to the preferential deposition of $A\beta_{42}$ in AD are conserved in the mouse. At 18 months of age, the percentage of shorter Aβ is essentially the same as at 12 months in both the cortex and hippocampus. Immunohistochemistry suggests that deposition of the $A\beta_{40}$ species is primarily in compacted plaques, as opposed to increasing amyloid angiopathy. The fact that the majority of Aβ detected in the mouse begins at Asp-1 is different from that reported in human AD (24). However, it is not clear how much of the amino-terminal modification in the human occurs after deposition. In AD, the plaques presumably have a residence time of several years, in contrast to several months, as in the case of the PDAPP mouse. The $A\beta_{42}$ found in the cerebrospinal fluid of AD patients is primarily $A\beta_{1-42}$ (unpublished data), suggesting the predominant cleavage sites are not shifted in the mouse from that in AD.

Aside from the insights into Aβ amyloidosis offered by the PDAPP model, there is a practical use of these studies as well. Using these measurements, it is now feasible to test agents that reduce Aβ peptide burden by preventing its production. Compounds designed to prevent or reverse Aβ deposition can also be evaluated in a reasonable *in vivo* fashion. Such agents, designed to prevent or reduce amyloidosis and plaque burden, will afford a new approach to the treatment of AD.

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ELSEVIER

NEW ADVANCES IN SOMATIC CELL NUCLEAR TRANSFER: APPLICATION IN TRANSGENESIS

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ABSTRACT

The ability to produce live offspring by nuclear transfer from cultured somatic cells provides a route for the precise genetic manipulation of large animal species. Such modifications include the addition, or "knock-in", and the removal or inactivation, "knock-out", of genes or their control sequences. This paper will review some of the factors which affect the development of embryos produced by nuclear transfer, the advantages of using cultured cells as donors of genetic material, and methods that have been developed to enrich gene targeting frequency. Commercial applications of this technology in biomedicine and agriculture will also be addressed.

Key words: nuclear transfer, nuclear reprogramming, cell cycle, transgenics, gene targeting.

INTRODUCTION

In the mouse, embryonic stem (ES) cells have provided an efficient method for genetic manipulation of the germ line and demonstrated the potential uses of this technology in a range of applications. Similar ES technology does not yet exist in farm animal species, however, the development of somatic cell nuclear transfer has bypassed the need for livestock ES cells. Successful somatic cell nuclear transfer using an embryo-derived differentiated cell population was first demonstrated in sheep in 1996 (10). Subsequently, the technique was repeated and extended using cell populations derived from fetal and adult donors in sheep (61). Furthermore, successful development has also been obtained in cattle (15, 25, 56, 62), goats (5, 26) and mice (51, 52). The production of offspring from differentiated cell nuclei provides information and opportunities in a number of areas including cellular differentiation, early development and genetic preservation, however, the primary significance of cloning is probably in the opportunities that this technology brings to the field of genetic manipulation.

This review will discuss methods available for the production of genetically modified mammals including recent progress and challenges associated with the production of transgenic animals via somatic cell gene targeting and nuclear transfer. Potential applications of this technology in agriculture and biomedicine will also be discussed.

Acknowledgments:

The authors gratefully acknowledge the helpful advice of David Ayares in the preparation of this manuscript.

Th riogenology 53:117–126, 2000
1999 by Elsevi r Sci nc Inc.

0093-691X/00/$–see front matt r
PII S0093-691X(99)00245-9

FACTORS AFFECTING EFFICIENCY OF NUCLEAR TRANSFER

The technique of nuclear transfer was originally proposed more than 60 years ago by Spemann (45) as a method to study cellular differentiation. However, it was almost entirely limited to amphibia until the early 1980's when McGrath and Solter demonstrated the possibilities of mammalian cloning (31). Subsequently in 1986, Willadsen (59) demonstrated that blastomere nuclei of sheep embryos at the 8-16-cell stage were competent to support full development after transfer into enucleated metaphase II (MII) oocytes.

The relative cell cycle status of the donor and recipient cell has been reported to be crucial to the success of nuclear transfer and the production of live offspring (6, 11, 17, 18). In mammalian species, enucleated MII oocytes have now become the preferred recipient, due to the lack of development obtained using enucleated zygotes (37, 35). When enucleated MII oocytes are used as recipients, the presence of high levels of maturation promoting factor (MPF) in the cytoplasm causes nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) of the donor nucleus following transfer. On subsequent activation of the oocyte, MPF activity declines, the chromatin decondenses, the nuclear membrane reforms, and DNA synthesis takes place. The effects of NEBD and PCC are dependent upon the cell cycle phase of the donor nucleus at the time of transfer. PCC of S-phase nuclei induces DNA damage, while the inappropriate occurrence of NEBD can result in uncoordinated DNA replication. When transferred at the time of activation, only nuclei that are diploid (G1/G0) at the time of transfer would appear to maintain the correct DNA content at the end of the first cell cycle. In contrast, when nuclei are transferred after the decline of MPF activity (pre-activated oocytes or zygotes), chromosomal damage induced by PCC is avoided and all nuclei, regardless of their cell cycle stage, undergo co-ordinated DNA replication. In addition to the effects of PCC and DNA replication, the cytoskeleton and mitotic spindle can also affect the ploidy, chromosomal constitution, and the subsequent development of the reconstructed embryos (reviews: 12, 14).

The cell cycle phase of the donor cell is also related to the developmental potential of reconstructed embryos. Quiescence has been implicated in the successful development of reconstructed embryos using somatic cell nuclei (10); however, the absolute requirement for quiescent cell nuclei in nuclear transfer is presently unresolved. The use of a diploid donor cell allows for coordination of donor and recipient cycles, while the use of MII oocytes as recipients maximizes the number of mitotic events that the donor chromatin undergoes prior to the initiation of zygotic transcription. The changes, which take place during cell quiescence, include a reduction in transcription, changes in the polyribosomes, active mRNA degradation and condensation of the nuclear chromatin (57). These changes may make the chromatin more amenable to respond to oocyte maternal cytoplasmic factors which control gene expression after nuclear transfer (for a review, see 14).

In the majority of reports to date, donor cells of embryonic, fetal and adult origin which were successfully used for nuclear transfer were either quiescent (G0) in situ (51), or were induced into G0 by serum deprivation. The results of nuclear transfer using somatic cells from different species are summarized in Table 1. Because the somatic cell nuclear transfer technique remains inefficient in terms of live births to initial number of reconstructed embryos, it is difficult to prove, in the absence of viable cell-stage-specific markers, from which cell donor,

quiescent or otherwise, an offspring was produced. Although several groups have reported live births using diploid fibroblasts from an actively growing culture (15, 52, 62), the question of whether the small proportion of the cells that developed to live animals were in the G1 or G0 phases is open. Boquest et al. (7) demonstrated that a primary population of actively dividing porcine fetal fibroblasts contains a sub-population of quiescent cells, and there is no reason to expect it to be different in primary cells of other species. In addition, sub-optimal culture conditions may increase the proportion of quiescent cells in the cell population.

If we consider the data reported in a range of species, we can see that successful development to term from transferable embryos varies significantly with a range from 1.0% to 83% (see Table 1). There are many factors that contribute to the development of reconstructed embryos. These include the quality of the recipient oocyte, method and time of activation, and culture methods. Similarly, induction and maintenance of pregnancy is also dependent upon a range of factors influenced both by the quality of the transferred embryos and the age, seasonality and hormonal status of the recipient. Therefore, it is difficult to conclude which factor or combination of factors contributed to making the cloning procedure more or less efficient in each of these reports.

GENETIC MODIFICATION IN MAMMALS

Pronuclear Injection

Transgenic animals can be successfully produced in a number of species including mice (24), rabbits, pigs, sheep, cattle and goats by the injection of the gene of interest into the pronucleus of a zygote (for a review, see 54). However, this technique suffers from several serious limitations (38, 60). The most profound limitation is that DNA can only be added, not deleted, or modified in situ. Also, the integration of foreign DNA is random, this could lead to erratic transgene expression due to effects at the site of incorporation. In addition, with random integration the possibility exists for the disruption of essential endogenous DNA sequences or activation of cellular oncogenes, both of which would have deleterious effects on the animal's health. Finally, transgenic animals generated using pronuclear microinjection are commonly mosaic, i.e., an integrated transgene is not present in all cells (40). Therefore, the production of the required phenotype coupled to germ line transmission could require the generation of several transgenic founder lines. Somatic cell nuclear transfer will eliminate this problem and accelerate transgenic herd or flock generation. In addition, transgenic sheep produced using this new technology required the use of fewer than half the animals needed for pronuclear microinjection (43).

Gene Targeting in Embryonic Stem Cells

Embryonic stem (ES) cells are derived from totipotent cells of early mammalian embryos and are capable of unlimited, undifferentiated proliferation in vitro (22, 30). The first examples of gene targeting in ES cells were at the hypoxanthine-guanine phosphoribosyltransferase gene (HPRT), a selectable locus located on the X chromosome (20, 49). Gene targeting of transgenes controlled both the site and copy number of the transgene insertion. The first report of germ line transmission from a targeted correction of a mutation in ES cells was also achieved at the HPRT

locus (50). Targeted mutagenesis of murine ES cells in culture followed by the production of germ-line chimeras has been widely used for over a decade as an important tool for introducing precise modifications of the germ line (33). A literature search revealed that over 800 published knockout mice strains have been generated (34).

Somatic Cell Nuclear Transfer

Until 1995 (9), the development of mammalian nuclear transfer reconstructed embryos was restricted to the use of donor genetic material from early embryos. Although this technique has a number of applications both on a scientific and a technological level it was the goal of many scientists to obtain development of nuclear transfer embryos from cells which could be maintained in culture. Many reports suggested that specific 'pluripotent' cell types (e.g., embryonic stem cells (ES) or primordial germ cells) would be required. Despite more than a decade of research focused on the isolation of ES cells in livestock species, such cell populations have as yet not been identified (for a review, see 13). The defining feature of the true ES cells, germ line transmission, has not yet been demonstrated using livestock ES-like cells (16, 36). However, the development of somatic cell nuclear transfer has bypassed the need for livestock ES cells. The somatic cell nuclear transfer system has an advantage compared with ES cell technology for producing transgenic animals, because the entire animal is derived from a single transgenic donor nucleus, thus eliminating the need for an intermediate chimeric generation before the effect of genetic modification can be assessed.

Gene Targeting in Somatic Cells

Early gene targeting experiments were carried out in transformed somatic cell lines such as mouse L cells (27), mouse-human hybrid cells (44), hamster CHO cells and human bladder carcinoma EJ cells. Few studies have shown that primary somatic cells can support normal absolute frequencies of gene targeting with no deterioration of morphology, karyotype or growth control (3). However, reports on gene targeting in somatic cells have been limited to rodent or human cells, and somatic cell gene targeting has only recently been reported for livestock (4, 58). Several behavioral differences between murine ES cells and primary somatic cells make gene targeting in somatic cells more difficult, including the apparent low frequency of homologous recombination and finite number of cell divisions. The frequency of homologous recombination in somatic cells is about two orders of magnitude lower than in ES cells, with very high frequencies of non-homologous recombination (53). To overcome this problem the targeting vector must provide a very powerful enrichment to suppress the appearance of non-homologously recombined clones. Three different enrichment methods have been developed: positive-negative selection, promoterless selection and polyadenylation signal-less selection.

1. In a positive-negative selection (PNS) vector, the positively and negatively selected genes are functionally independent expression cassettes and each contains its own promoter and polyadenylation signals (42). The positive selection marker selects only for those cells that have stably incorporated the vector, but does not differentiate between random or homologous events. Negative selection results in the selective killing of cells that have undergone recombination by a nonhomologous event, thus enriching for events that were

the result of homologous recombination. PNS vectors usually achieve enrichments by only 2 to 5-fold (29).

2. Promoterless resistance markers express only if integrated properly downstream of a promoter active in the cells. Some non-homologous recombination events can still be recovered if the vector integrates close to some chromosomal promoter that allows sufficient expression to obtain a drug-resistant clone. However, these events are quite rare. Therefore, this promoter-trap technique typically achieves enrichments of 100 to 500-fold. Such powerful selection, while not necessary in ES cells because of their high recombinogenicity, is absolutely essential for efficient gene targeting in somatic cells.
3. Polyadenylation signal-less markers produce stable transcripts only if inserted upstream of a genomic polyadenylation signal. The poly-A trap technique produces a higher level of enrichment than PNS vectors but lower than that obtained by the promoter-trap method.

An important criterion for choosing a cell line is that it should have a high single-cell cloning efficiency because during drug selection, single cells must be capable of expansion into clonal cultures. Theoretically, 21 population doublings should be enough to generate more than $2x10^6$ cells through clonal expansion. The life span for porcine and bovine fetal fibroblast cells ranges between 30-50 population doublings. However, the single cell cloning efficiency for most primary (non-immortalized) cells is very low. For example, human dermal fibroblasts, when seeded as single cells, are not able to proliferate under regular culture conditions (23). Therefore, optimization of culture conditions for single cell clone isolation is critical for the success of gene targeting in somatic cells.

Gene Targeting in Livestock Cells

A high frequency of homologous recombination (HR) has been achieved in ovine primary fetal fibroblast cells (4). The frequency of targeted insertion of a selectable marker gene was 9.2% (11 positive colonies out of 119 screened). In a separate experiment, a commercially important transgene, alpha-1-antitrypsin (AAT), was successfully inserted, along with a marker gene, via homologous recombination in sheep fetal fibroblast cells; the frequency of an AAT targeted insertion was 66% (46/70). Nuclear transfer using targeted fibroblasts as nuclear donors was performed as described previously (10) and resulted in the births of Cupid and Diana (July 1999) – the first non-mouse gene-targeted animals to be produced (see Table 1). The generation of these gene-targeted farm animals demonstrated, for the first time, that homologous recombination technology, which was well documented in mouse ES cells, is now available for livestock species via somatic cell nuclear transfer (58).

APPLICATIONS OF NUCLEAR TRANSFER TECHNOLOGY

The demonstration that live offspring can be obtained from embryonic, fetal and adult derived cultured cell populations has not only answered one of the fundamental questions of developmental biology but also provided potential applications in a wide range of fields including numerous areas of basic research and animal biotechnology (for a review, see 19). Here we will discuss some applications of this technology, specifically those that require precise

genetic modification of farm animal species by gene targeting, and the role of nuclear transfer in cellular dedifferentiation.

Potential Applications of Gene Targeting in Large Animal Species

Previously, the ability to perform precise genetic modifications in mammals was restricted to mice (28). The births of Cupid and Diana, the first farm animals produced by nuclear transfer using targeted somatic cells, removed this impasse (58). The benefits of this technology are numerous. For example, it will allow a gene of interest to be inserted at a specific chromosomal site, chosen for its ability to facilitate high expression from any inserted gene. This ability to enhance expression will significantly increase the capacity to produce therapeutic and nutritional proteins in the milk of transgenic livestock.

Gene targeting is likely to play a major role in preventing hyperacute rejection (HAR) in organ xenotransplantation. HAR is the initial and most dramatic response to pig vascularized organs. This is known to be triggered by pre-formed antibodies binding to the endothelium lining the blood vessels of the pig organ. The bound human antibodies rapidly activate the complement cascade, as well as the endothelium, inducing it to become pro-coagulatory. The result of this is total destruction of the graft within minutes to hours of transplant. Evidence has emerged in the last decade that this is due to a carbohydrate epitope, galactose linked via an α $(1\rightarrow3)$ linkage to a second molecule of galactose, to which about 1% of human immunoglobulins crossreact (41). The high levels of circulating antibodies to this structure are thought to form a first line of defense against pathogens that express the carbohydrate.

The most direct method of preventing the adverse HAR response involves the production of donor animals with an α-1,3 galactosyl transferase (GT) gene knockout. Removal of this enzyme activity would lead to the total lack of expression of α-1,3 GT at the cell surface and this should dramatically reduce HAR. However, there are two problems with this approach. Firstly, at the present time there have been no reports of cloned pigs produced via somatic cell nuclear transfer. This is possibly due more to the technical and physiological issues relating to oocyte activation, embryo culture, induction and maintenance of pregnancy than somatic cell reprogramming per se. Only a single pig has been successfully cloned using an early blastomere as the nuclear donor (37). Secondly, even though homozygous knockout (α-1,3 GT, -/-) is not lethal in mice (47, 48), the galactose α $(1\rightarrow3)$ galactose structure may provide some essential biological function in pigs; thus, destroying the α-1,3 GT enzyme could be deleterious to the animals.

Other potential candidates for gene targeting in farm animals include:

1. Replacing bovine serum albumin with the human form thus allowing the cost-effective production of human serum albumin in the milk of cows.
2. Knockout of the Prion gene involved in spongiform encephalopathies of sheep (scrapie) and cows (BSE). This gene was successfully inactivated in mice without any deleterious effect (8). Weissmann et al. (55) demonstrated that homozygous PrP knockout mice remained free from scrapie for at least two years after inoculation with prions while wild-type controls all died within six months.

In addition to their roles in agriculture and animal biotechnology, livestock species, especially pigs, cows and sheep, are often better models for human physiology and disease than rodents because of their similarities in anatomy, physiology and size (35). For example, the pig could be a well-suited model for human eye diseases such as retinitis pigmentosa, because of the similarity in eye size and retinal anatomy (1). Sheep may be ideal candidates to generate a model for human cystic fibrosis by targeting a mutation to the cystic fibrosis gene. However, due to the previously low efficiency of transgenic livestock production, and a lack of availability of homologous recombination techniques, its application has been very limited in the past. Nuclear transfer technology will allow the production of disease models in species which are physiologically more similar to the human thus allowing one to better follow disease progression and to assess the benefits of any potential new therapies.

Trans-Species Nuclear Transfer

The demonstration that the recipient cytoplast has the ability to reprogram the chromatin of adult somatic cells of different species (21, 32) provides a wide range of potential clinical applications. Use of the trans-species nuclear transfer technique may allow the generation of human embryonic stem cells from a somatic cell of a patient, following fusion with a bovine enucleated oocyte (2). The use of enucleated bovine oocytes would bypass some of the ethical issues associated with the use of excess human IVF embryos for generating human therapeutic cell lines. This advance may enable the production of immunologically matched stem cells, which could then be differentiated in culture to specific phenotypes. Such cells may then be returned to the patient avoiding the problems of rejection due to mismatching of tissue types. Such cells could be used to replace lost cell populations which resulted from nerve damage or Parkinson's disease (63), possibly as a treatment for immune disorders, leukemia and other blood diseases or the cells could be used as vehicles for gene therapy.

CONCLUSIONS

The utilization of nuclear transplantation for livestock species promises to provide enormous benefit to biotechnology, biomedicine, farm animal breeding and research. However, the efficiency of this procedure is still low in relation to pregnancy and development-to-term rates, which are significantly lower than those resulting from in vitro-produced embryos. In addition, a significant proportion of the offspring produced by somatic cell nuclear transfer exhibit a variety of abnormal symptoms that often result in the death of the animals shortly after birth. The mechanism of somatic cell nuclear reprogramming, the effect of karyoplast source, and its differentiation on reprogramming as well as potential species-specific differences are still unknown. While it may be acceptable to use this technology even with its current limitations, for applications related to biotechnology, a lot of challenges will need to be overcome before this technique will be useful on a broad scale for agricultural applications.

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Special Review Series – Gene Manipulation and Integrative Physiology

Germ line transformation of mammals by pronuclear microinjection

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The most popular approach for generating transgenic mammals is the direct injection of transgenes into one pronucleus of a fertilized oocyte. In the past 15 years microinjection has been successfully applied in laboratory as well as in farm animals. The frequency of transgenic founders, although highly different between the species, is efficient enough to render this technique applicable to a wide range of mammals. The expression levels and patterns of a transgene are initially influenced by the construction of the transgene. However, the overall phenotype of a transgenic organism is influenced by several genetic and environmental factors. Due to the features of this technique not all of the genetic factors can be experimentally controlled by the scientist. In this article we will emphasize some peculiarities which have to be taken into account for the successful performance of transgenesis by pronuclear microinjection. *Experimental Physiology* (2000) **85.6**, 589–601.

Introduction: methods of transgenesis

In 1981 Gordon and Ruddle introduced the term 'transgenic' in relation to mice genetically modified by transmission of foreign DNA into early embryos. Today it is applied to the characterization of an animal whose genome has been altered by the stable integration of an *in vitro* recombined genetic sequence.

Three different methods are routinely used to generate transgenic animals: (i) transfection of early embryos with recombinant retroviruses carrying the gene of interest, (ii) pronuclear microinjection of DNA into a zygote and (iii) gene transfer into pluripotent embryonic stem (ES) cells, originally isolated from the inner cell mass of a blastocyst.

Recently, a fourth method has been introduced. The transmission of foreign DNA was successfully performed by intracytoplasmatic coinjection of unfertilized mouse oocytes with sperm heads whose membranes had been disrupted (Perry *et al.* 1999). If reproducible, this procedure has a potential as a method for transgenesis in the future.

The aim of transgenesis is the establishment of a transgenic line. Because of the early differentiation between soma and germ line in mammals, genetic experiments have to be carried out as early as possible during ontogenesis. Therefore, pre-implantation stage embryos are preferentially used (Rülicke, 1996).

Both infection with retroviral vectors and microinjection of naked DNA are restricted to the addition of DNA sequences, whereas gene targeting in cultured ES cells can alter an endogenous gene by homologous recombination of a chromosomal locus with the transfected DNA molecule. Depending on the field of application each of these methods has its advantages as well as limitations.

The importance of retroviral vectors for the generation of transgenic animals has decreased markedly and was gradually replaced by pronuclear microinjection since these vectors are limited to about 8 kb of foreign DNA. Furthermore, particular viral sequence motifs appear to suppress the expression of transgenes and the retroviral vectors are associated with a certain biohazard risk. Nevertheless, retroviral vectors have been used in avian species where the generation of transgenic animals through microinjection of naked DNA is particularly difficult and not yet routinely applicable.

The ES cell technique, although of great interest in other model organisms and in livestock species, has been successfully used only in the mouse so far. Plenty of very valuable mouse mutants have been generated by this approach. They include either a loss-of- or a gain-of-function mutation. Recent developments in nuclear transfer technology could provide an alternative tool to circumvent the lack of ES cells in other mammalian species.

Publication of *The Physiological Society*

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The most popular approach for generating transgenic mammals is direct injection of transgenes into one pronucleus of a fertilized oocyte. In the last 15 years microinjection has been successfully applied in laboratory as well as in farm animals. The frequency of transgenic founders, although highly different between the species, is efficient enough to render this technique applicable to a wide range of mammals.

In this article, we consider only pronuclear microinjection. We will emphasize some peculiarities, which have to be taken into account for the successful performance of transgenesis by this technique.

Pronuclear microinjection of mouse oocytes

Fertilized oocytes for microinjection are recovered from the oviduct of mated female mice after superovulation. The timing of development through the first cell cycle is shown in Fig. 1.

Mouse pronuclei are easily visible during the S-phase, the time frame when the first round of DNA replication takes place. The early female pronucleus is located not far from the second polar body where meiosis was completed after fertilization (Fig. 2*A*). About 6 h later both pronuclei show increased size and are centrally positioned (Fig. 2*C*). The size and visibility of both pronuclei are mainly influenced by the oocyte, i.e. by the genotype (strain) of the female. For microinjection the male pronucleus is preferred because of its larger size and better position (Fig. 2*B*).

The two pronuclei differ in their DNA structure. In oocytes, the maternal genome is packaged into chromatin. The paternal genome arrives packaged in protamines that are replaced with histones provided by the egg after fertilization (Zirkin *et al.* 1989). However, there is apparently no difference in the number of transgenic offspring after injection into male or female pronuclei (Brinster *et al.* 1985).

For the microinjection the zygotes are immobilized with a holding pipette (Fig. 2*B*). The oocytes which survive the injection procedure (about 40–90%, depending on the occupational skill) are transferred into the oviduct of a pseudo-pregnant surrogate mother. In our hands, about 20% of the transferred embryos develop to term and more than 20% of these live-born offspring are transgenic founders resulting in an overall yield of 1.6–3.2%.

Integration characteristics of the pronuclear injected transgenes

During microinjection about 1–2 pl of DNA solution are transferred into one pronucleus. The injection buffer typically contains 2 ng DNA μl^{-1}. Depending on the size of the transgene each zygote receives tens to several hundreds of linearized DNA molecules. Before chromosomal integration takes place, the injected DNA molecules recombine to large tandemly arranged concatemers. Although the dominant intermolecular recombination mechanism in mammalian cells is non-homologous, the predominant head-to-tail array strongly suggests a process of homologous recombination between multiple copies (Brouillette & Chartrand, 1987; Bishop & Smith, 1989).

The integration of pronuclear injected DNA was demonstrated by *in situ* hybridization in diverse regions of the mouse genome (Michalova *et al.* 1988; Festenstein *et al.* 1996; Dobie *et al.* 1996; Boyer *et al.* 1997). These observations supported the assumption that integration sites of transgenes are randomly distributed at unreproducible chromosomal sites. Homologous recombinations of pronuclear injected constructs with an endogenous locus is extremely infrequent (Brinster *et al.* 1989).

Only 13.2% of all founders produced in our laboratory had multiple unlinked insertions in more than one chromosomal site, suggesting that the integration of transgenes is determined by rare nuclear events. It seems reasonable to assume that illegitimate end-joining after DNA strand breaks is the main route of randomly distributed chromosomal integration of pronuclear injected transgenes.

It is quite possible that the procedure of microinjection itself could induce random chromosomal damage leading to preferentially used integration sites. In addition to random DNA strand breaks, the activity of DNA topoisomerase I may play a role in the insertion process (Konopka, 1988). As a result of so-called non-homologous or illegitimate recombination there exists probably more than one mechanism of transgene integration.

Microinjected DNA can also persist for several days as free molecules. The distribution of foreign DNA between the blastomeres of an embryo was not found to be due to early

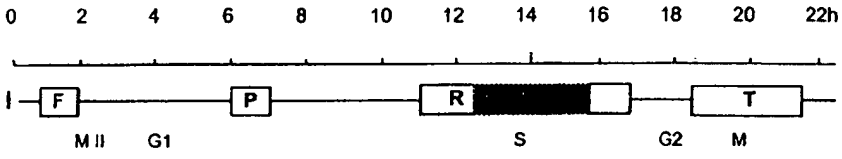

Figure 1

The first cell cycle of mouse development after insemination. Insemination was 13.5 h post hCG-injection for superovulation (Howlett & Bolton, 1985). I, insemination; F, *in vitro* fertilization; P, start formation of pronuclei; R, DNA replication; ■, time period for microinjection; T, first cell devision; M II, meiosis II; G, gap phase (either G1 or G2); S, DNA synthesis; M, mitosis.

concatemerization (Cousens *et al.* 1994). Consequently, there is ample opportunity for an integration event in single blastomeres after the first round of DNA replication. Indeed, in our laboratory almost 7.6% of all founders were genetic mosaics without germ line transmission while 31.1% were mosaics with germ line transmission (Rülicke & Mertens, 1999).

Non-homologous recombination of microinjected DNA can cause severe genetic alterations in the flanking chromosomal regions (Covarrubias *et al.* 1986; Wilkie & Palmiter, 1987; Kohrman *et al.* 1995). Such rearrangements include deletions, duplications and translocations. This is directly opposed to the integration of retroviral vectors which merely induce a duplication of four to six base pairs of the flanking sequence (Coffin, 1996).

Inheritance and expression of pronuclear injected transgenes

Stable integrated transgenes are usually transmitted to the offspring in a Mendelian fashion. Variations of the expected ratio can be caused by founders containing mosaic germ line or multiple integration sites. Furthermore, X- or Y-chromosomal integrations, detrimental insertional mutations or harmful effects of the transgene product can affect the pattern of inheritance.

The activity of transgenes can be measured at the levels of mRNA (by Northern blot analysis) and protein (by immunoblots or functional analysis) or by physiological effects of the transgene product itself. However, the overall phenotype of a transgenic organism is influenced by several genetic and environmental factors. Not all of these genetic factors shown in Fig. 3 can be experimentally controlled by the scientist, implicating that each founder line, even if harbouring an identical transgene, is unique and has to be characterized separately.

The expression levels and patterns are heritable properties of a transgenic line. Both are initially influenced by the construction of the transgene, such as the correct use of regulatory and/or coding sequences.

The construction of transgenes

In higher eukaryotes the regulation of gene expression is a very complex macromolecular event. Although the expression pattern of transgenes may be influenced by post-transcriptional and post-translational processes, the regulation of transcription *per se* is the most stringent way to control gene expression. Gene activation and the rate of transcription depend on different transcriptional control elements. Furthermore, some of these *cis*-factors regulate the correct spatial and temporal activity of a (*trans*-) gene, suggesting that each gene has a

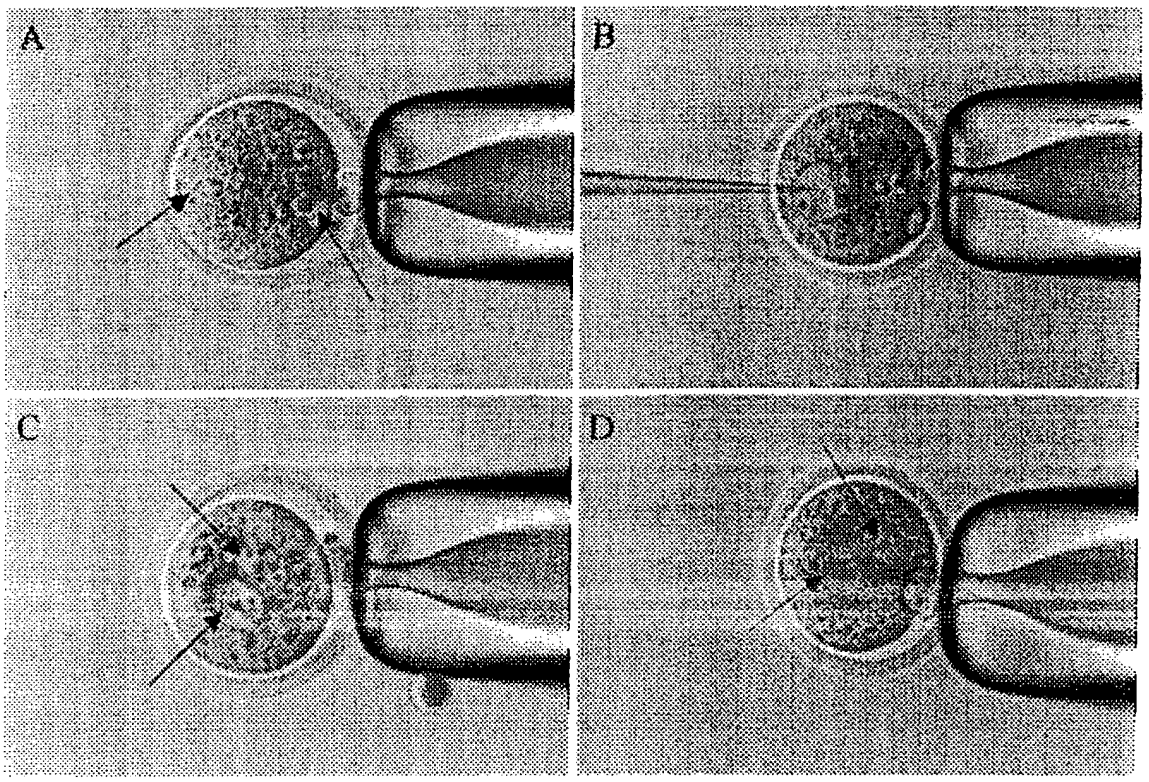

Figure 2

Development of pronuclei and microinjection of DNA into the mouse zygote. *A* and *C*, development of pronuclei (arrows), about 20 and 26 h post hCG-injection for superovulation; *B*, DNA-microinjection into the male pronucleus; *D*, pronuclei disappear before the first cell division.

unique arrangement and assortment of control elements. Results of transgenic experiments suggested that the *cis*-regulatory elements co-operate among each other and that the complete set of a particular gene locus must be present for an integration site-independent expression (Huber *et al.* 1994).

Transcription control elements of eukaryotic protein coding genes are found in the core promoter and in other upstream/downstream regulatory sequence motifs. Regulatory sequences can function as binding sites for gene-specific transcription factors, which can stimulate transcription directly through interactions with components of the basal transcription initiation complex (RNA polymerase II holoenzyme and the basal transcription factors) or indirectly by preventing the repressive effects of a closed chromatin structure.

The best characterized control element so far is the promoter located immediately upstream of the transcription initiation site. But a promoter alone is often unable to express a transgene. This is frequently observed in transgenic mice carrying a simple cDNA construct.

A second type of control element, the enhancer, is a DNA sequence which stimulates transcription. An enhancer can potentiate gene activity in a tissue-specific manner by binding activating transcription factors (reviewed by Müller *et al.* 1988).

Mice of different founder lines usually show qualitatively and quantitatively different patterns of gene expression. These so-called position effects due to random sites of transgene integration could be eliminated for transgenes harbouring a *cis*-regulatory element known as the dominant control region (DCR) or locus control region (LCR) (Grosveld *et al.* 1987; Bonifer *et al.* 1990). LCRs are short stretches of DNA, characterized by tissue-specific developmentally stable DNase-I hypersensitive sites which are able to provide an open chromatin structure to adjacent sequences even when the transgene is integrated into heterochromatic centromere regions of mouse chromosomes (Festenstein *et al.* 1996). LCRs have been identified, for example, upstream of the globin gene cluster and downstream of the CD2 gene (Orkin, 1990; Lake *et al.* 1990). The chromatin opening activity and insulating properties of LCRs may additionally be influenced by certain other gene components, not always present in cDNA- and hybrid constructs (Reitman *et al.* 1993). The β-globin LCR for instance was incapable of conferring position-independent expression onto the prokaryotic marker sequences of lacZ (Guy *et al.* 1996). The effect of LCRs can also be influenced by an increased copy number indicated in mice as age-dependent silencing of transgene expression (Robertson *et al.* 1996).

Several other specific control elements have been described that can improve the expression of transgenes. Matrix/scaffold attachment regions (MARs/SARs) are thought to be important for the organization of chromatin into functional genetic domains as a consequence of binding to the nuclear matrix (Bode *et al.* 1992). Positive effects of MAR elements onto accurate position-independent and copy number-dependent expression have been reported for transgenic mice (McKnight *et al.* 1992). Subsequent investigations, however, indicated that the positive effects of MARs on transgene expression are not universal but rather depend on the co-operation with other regulatory elements (Thompson *et al.* 1994; Barash *et al.* 1996; Neznanov *et al.* 1996).

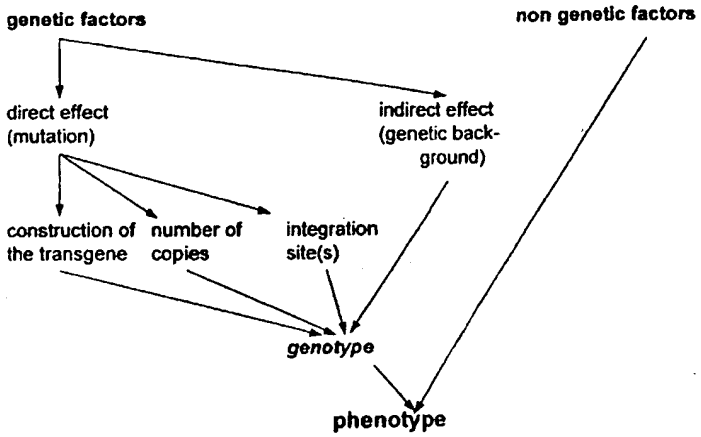

Figure 3

Factors that influence the phenotype of a transgenic animal. The phenotype of an animal is the result of different genetic and non-genetic factors. Besides the background genome, direct effects of the mutation, such as the included regulatory non-coding as well as coding sequences, the integration site(s) and the number of integrated copies of a transgene, are important. Non-genetic factors are, for instance, the housing conditions, the husbandry during experiments and the microbiological environment.

Chung *et al.* (1993) described a novel regulatory element near the 5' boundary of the chicken β-globin gene. This element insulated transgenes in *Drosophila* and mice without accompanying activation or suppression effects (Chung *et al.* 1993; Wang *et al.* 1997). More recently, Siegfried *et al.* (1999) have characterized an element from the CpG island sequence (unmethylated CpG-dinucleotide rich segment of housekeeping genes) of the hamster *APRT* locus that may improve transgene expression due to the reduced *de novo* methylation of flanking sequences up to 150 bp away.

Finally, two technical points have to be taken into account during the construction and preparation of linear transgenes for pronuclear injection. Firstly, prokaryotic sequences of the cloning vector have to be removed from the transgene (Townes *et al.* 1985; Shani, 1986). Secondly, the injected linear constructs are prone to nuclease degradation. If a sequence loss of more than 100 bp is observed non-essential flanking regions have to be added to both sides of the construct (Kearns *et al.* 1995).

cDNA or genomic sequences as source for transgenic constructs. A comprehensive knowledge of the specific gene is required in order to provide defined transgene expression in a quantitative and qualitative manner. For many interesting candidate proteins only the cDNA sequences but not the specific regulatory elements are currently available. Therefore, in transgenes using cDNA, the promoter of the gene to be mimicked is routinely combined with a heterologous enhancer, introns and polyadenylation signals. Such a transgene often results in an unpredictable expression level. Furthermore, tissue- or developmental stage-specific isoforms of (*trans*-) gene products may be the result of alternative splicing, a process that cannot occur when cDNA constructs were used for microinjection.

In experiments for the reconstitution of the knockout mouse for the prion protein (PrP) we have injected three different constructs of the murine PrP coding locus as transgenes (Fischer *et al.* 1996). Transgenic mice harbouring 40 kbp of the complete genomic sequence including both introns and the flanking regions showed the expected PrP expression. To facilitate mutagenesis, a half genomic PrP minigene was constructed from which the large downstream intron and the downstream flanking region had been deleted (Fig. 4).

In all but one of six lines carrying this construct, PrP was correctly expressed. However, different levels of expression between the founder lines indicated a stronger sensitivity against position effects of the half-genomic construct. In contrast, all eight transgenic lines containing the cDNA construct had no detectable levels of PrP. Although we cannot exclude the loss of an unknown regulatory sequence with the upstream intron, the missing expression of the cDNA is probably due to the general lack of introns. A requirement of introns for efficient transgene expression *in vivo* has been described previously (Brinster *et al.* 1988; Whitelaw *et al.* 1991; Palmiter *et al.* 1991). Introns can improve expression of transgenic constructs by different mechanisms. (i) Through *cis*-acting control elements within intron sequences. (ii) The correct splicing process might influence transgene expression through an enhanced mRNA stability. (iii) The introduction of a generic intron that consists only of an splice donor and a splice acceptor site stimulates the expression of a transgene compared with the construct without any intervening sequence (Choi *et al.* 1991). (iv) The loss of introns in cDNA constructs might lead to an unnatural sequence that effects chromatin structure, i.e. the nucleosome arrangements that may prevent transcription initiation or elongation (Liu *et al.* 1995).

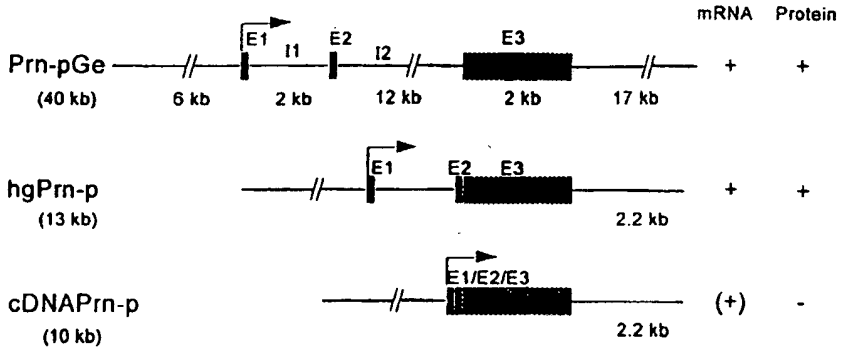

Figure 4

Constructs encoding wild-type PrP for reintroduction into Prn-p^{tm1} knockout mice. E1–3, exons; I1–2, introns; + or –, PrP expression of transgenic mice monitored by Northern analysis (mRNA) or Western blots (protein) of total mouse brain homogenates. The cDNA construct showed no detectable levels of prion protein and no or only very weak expression of Prn-p mRNA (+).

Most interestingly, in mice containing the half-genomic PrP-minigene we found no PrP mRNA in Purkinje cells, PrP mRNA is normally abundant in wild-type as well as in PrP knockout animals that have been reconstituted with the genomic sequence. This suggested that a control element which is essential for Purkinje cell-specific PrP expression was lost within the half-genomic minigene (deletion of the 12 kbp intron and the 3′ flanking region).

The take home message from this work is to employ genomic constructs for generation of transgenic animals whenever possible. This should be followed by the construction of (half-) genomic minigenes in which large intronic regions are removed to facilitate transgenesis.

Other strategies were also developed for construction of minigenes in order to circumvent the problems related to cDNA. Moreover, for an ectopic, conditional or inducible expression of transgenes the coding region must at least be coupled with heterologous regulatory sequences.

The leakage of essential regulatory elements and the necessity of introns for RNA splicing to produce stable RNA can, for example, be solved with the insertion of the cDNA into a complete or incomplete heterologous genomic locus. In this way the viral *src*-kinase and the human Foamy virus *env*-gene were expressed in a tissue-specific manner after cloning the cDNA into the first exon of the genomic *Gfap*-sequence (Weissenberger *et al.* 1997; Lampe *et al.* 1997). The risk of this strategy is that artificial exons are skipped (Müller *et al.* 1994; Davisson *et al.* 1996) or that cryptic splice sites in large exon sequences are used (reviewed by Berget, 1995). Such constraints can be bypassed with the use of an internal ribosomal entry site (IRES). Such sequences are provided by viruses from the picorna family. IRES sequences act as a ribosome landing pad and mediate the translation of di- as well as polycistronic constructs (Pelletier & Sonenberg, 1988). This strategy allows the cDNA to be preferably positioned in the 3′ untranslated region of an intact gene and lowers the risk of skipping (Kim *et al.* 1992; Mountford *et al.* 1994; Vaulont *et al.* 1995). However, since the IRES sequences are of viral origin they could become the target of host defence mechanisms.

Cloning and microinjection of large DNA fragments. If the intention is to express a transgene in its native pattern the best approach is to transfer the gene as an entire genomic locus. Since many eukaryotic genes span large regions of DNA, it excludes the use of traditional cloning vectors and standard microinjection procedures. Large transgenes can be cloned as overlapping DNA fragments and coinjected in equimolar amounts. The functional transgene can be efficiently reconstructed by homologous recombination between the DNA molecules after injection (Pieper *et al.* 1992; Keegan *et al.* 1994). Up to 300 kbp DNA fragments can be cloned with bacteriophage P1 artificial chromosomes (PACs) (Ioannou *et al.* 1994) or bacterial artificial chromosomes (BACs) (Shizuya *et al.* 1992). Very large genomic loci extending several hundreds of kilobase pairs can be handled via yeast artificial chromosomes (YACs) and transferred under special conditions by pronuclear injection (Burke *et al.* 1987; Gnirke *et al.* 1993; Schedl *et al.* 1993*a*,*b*). The small aperture of the micro-injection needle (< 1 μm) causes likely shearing for large DNA molecules. Low concentrations of polyamines in combination with high salt concentrations can protect DNA from shearing (Montoliu *et al.* 1994; Mrkic *et al.* 1998).

The transmitted number of YAC molecules per injected oocyte is very low compared with injection of traditional transgenes. A DNA concentration of higher than 10 ng (μl injection buffer)$^{-1}$ may lead to a reduction in survival of the injected zygotes (Brinster *et al.* 1985). Nevertheless, the resulting frequencies of transgenic offspring after YAC injection varies between 5 and 21 % and is only slightly lower than after injection of hundreds of small constructs (Schedl *et al.* 1993*a*,*b*; Peterson *et al.* 1995, 1996; Heard *et al.* 1996; Mrkic *et al.* 1998).

Position effects on transgene expression

The expression of pronuclear injected transgenes is often highly variable between different lines containing an identical construct. This is mainly due to the random integration sites. The position effects can be caused by integration into, for example: the direct vicinity of transcription control elements of a host gene, a region of imprinting, a non-transcribed heterochromatin region and X- or Y-chromosomes.

Transcription interference and insertional mutations after random integration. The effect on transcription by *cis*-acting endogenous regulatory DNA sequences has been demonstrated by spatial and temporal variable expression patterns in different transgenic lines with randomly integrated marker genes (Kothary *et al.* 1988; Allen *et al.* 1988). Position effects are exerted most strongly on those transgenes with tightly controlled promoters and that do not contain further regulatory sequences such as an enhancer (Müller *et al.* 1988), a locus control region (Grosveld *et al.* 1987; Palmiter *et al.* 1993) or a CpG island element (Siegfried *et al.* 1999). Al-Shawi *et al.* (1990) demonstrated the influence of the random integration site by the isolation of a poorly expressed transgene locus from a mouse line. After reintegration of the isolated array, the secondary transgenic mice showed the expected expression level comparable to other transgenic lines harbouring the identical construct.

Although difficult to prove, it is also conceivable that the activity of endogenous genes could be influenced by proximate transgenic loci. Therefore, in a knock in approach the selection marker of the targeting construct has to be removed after homologous recombination.

Randomly integrated transgenes can induce insertional mutations in endogenous genes, amplified by severe alterations of flanking chromosomal regions. Although several thousand transgenic lines have been published, the number of reported insertional mutants is very low so far (reviewed by Rijkers *et al.* 1994). Taking into account that only viable and fertile mutants causing strong phenotypes were identified in characterized lines, the real frequency of insertional mutations should clearly be higher.

Genomic imprinting and *de novo* methylation f integrated transgenes. In a minority of transgenic lines, an epigenetic mechanism known as genomic imprinting can be observed in hemizygous animals (Reik *et al.* 1987; Swain *et al.* 1987; Sapienza *et al.* 1989; De Loia & Solter, 1990; Sasaki *et al.* 1991; Fafalios *et al.* 1996). A reversible gamete-specific modification can lead to functional differences between maternal or paternal inherited transgenes in hemizygous offspring. Although many chromosomal regions of the mouse genome are involved in genomic imprinting, imprinted transgenes could so far never be exclusively attributed to a positional effect.

An outstanding characteristic of transgene imprinting is the almost complete restriction of transgene inactivation to the female germ line and the influence of modifiers of the genetic background (Sapienza *et al.* 1989; Engler *et al.* 1991; Chaillet *et al.* 1991). In contrast to endogenous loci, where imprinting takes part in the regulation of gene expression, the inactivation of transgenes could be a host defence mechanism against foreign DNA.

The differential imprinting of alleles of specific genes in the human or mouse genome has been correlated with DNA methylation (Li *et al.* 1993). Interestingly, the inactivation of transgenes by *de novo* methylation has frequently been observed in stable transfected cells and in transgenic mice. Constructs containing viral sequences are especially susceptible to this process (Palmiter *et al.* 1982; Wienhues & Doerfler, 1985; Koetsier & Doerfler, 1996; Betzl *et al.* 1996). This observation supports the hypothesis that *de novo* DNA methylation of transgenes can also be interpreted as a defence mechanism against the activity of foreign genes in an established genome.

The influence of heterochromatin and position effect variegation on transgene expression. In mouse chromosomes, A–T rich satellite DNA is localized in heterochromatin near the centromere. Pericentric heterochromatin can be visualized as a C-band and is characterized as a late replicated and transcriptional inactive region. The capability of heterochromatin to inactivate translocated euchromatic sequences has been termed position effect variegation (PEV). Primarily observed and investigated in *Drosophila* (reviewed by Weiler & Wakimoto, 1995), PEV is also present as position effect in transgenic mice. Transgene expression is usually assumed to be uniform in specific cells of a tissue. But cell-by-cell analysis of transgene-encoded molecules has shown that even in mice of an established line a mosaic pattern of transgene expression exists (Elliott *et al.* 1995; Festenstein *et al.* 1996; Dobie *et al.* 1996; Boyer *et al.* 1997). Fluorescence *in situ* hybridization analysis of transgene integration sites indicates that this phenomenon is observed when the transgene is located in the vicinity of pericentric heterochomatin. This position-dependent effect results in the stochastic inactivation of transgene expression in a subpopulation of cells of a particular tissue. Furthermore, the percentage of expressing cells declines with the increase in copy number within the integrated array (Dorer & Henikoff, 1994).

Consequences of X- and Y-chr mosomal integration. Sex chromosomal integration of a transgene leads to special features regarding its inheritance and expression. The analysis of Y-chromosomal integrated transgenes is restricted to the male gender. This most specialized mouse chromosome consists predominantly of highly repeated DNA sequences with no obvious function (Bishop, 1993). Additionally, because of its extremely late replication during mitosis, characteristic of heterochromatic segments, an optimal expression of transgenes on the mouse Y-chromosome may not be expected (Pravtcheva *et al.* 1994).

X-chromosomal integrated transgenes can be subjected to modifications similar to those reported for endogenous X-linked genes. The inactivation of one X-chromosome in female mammals occurs with different schedules in different tissue lineages of the conceptus. In female extraembryonic tissues, the paternally derived X-chromosome (including the transgene) was preferentially inactivated by 3.5–4.5 days postconception. In somatic cell lineages the X-inactivation in most tissues was completed by 9.5–10.5 days postconception. Hemizygous female embryos displayed a mosaic pattern of transgene expression in about 50% of the somatic cells, most probably due to random inactivation of one X-chromosome per cell (Tagaki & Sasaki, 1975; Tan *et al.* 1993). However, it has also been reported that a transgene inserted into the X-chromosome might completely escape X-chromosome inactivation (Goldman *et al.* 1987). This could be due to integration in the X–Y pairing region, which normally escapes X-inactivation.

Multicopy array and its influence on transgene expression

The copy number of transgene concatemers varies usually between one and about 100 (Fischer *et al.* 1996; Magyar *et al.* 1996; Weissenberger *et al.* 1997; Raeber *et al.* 1999). However, an extreme tandem array of 1000 copies spanning an insertion of 11000 kbp has been described (Lo *et al.* 1992; Simon & Knowles, 1993). There is usually no correlation found between the copy number and the expression level of transgenes. However, for transgenes harbouring LCRs, a copy number-dependent expression level was observed (Grosveld *et al.* 1987; Bonifer *et al.* 1990). These results indicated that each transgene copy can function as an independent regulatory unit.

Silencing of transgenes in animals with high copy number is a frequent feature of pronuclear microinjection. Large tandem arrays of repeated sequences may repress efficient transcription of transgenes since inappropriate chromatin structures were induced (Simon & Knowles, 1993; Dorer & Henikoff, 1994). A twenty- to fiftyfold reduction post integration of a high copy number array (from more than 100 to five, respectively one copy) in two independent mouse lines resulted in a marked increase in the expression level of the respective transgene. Simultaneously, chromatin compaction and DNA methylation at both recombined transgene loci were decreased (Garrick *et al.* 1998).

Changes in chromatin structure after copy number reduction suggest that the reduced transcription rate per integrated

transgene is not primarily due to the limitations of essential transcription factors. This also became clear by an increased gene expression level observed in several homozygous and hemizygous transgenic mice (Fischer *et al.* 1996; Senn *et al.* 2000).

Another form of (*trans*-) gene silencing that was unexpectedly discovered in plants, fungi and, more recently, in *Drosophila*, results from the interactions between homologous genes (Cogoni *et al.* 1996; Metzlaff *et al.* 1997; Pal-Bhadra *et al.* 1997). By this interaction, which is called co-suppression, a transgene can inactivate its endogenous counterpart or an identical transgene that is integrated at another genomic location. Co-suppression-associated gene silencing is most probably induced by several mechanisms. Transcriptional repression in conjunction with DNA methylation and repressive chromatin structure, as well as post-transcriptional silencing by elimination of RNA products have been described in several cases (Matzke & Matzke, 1995; Baulcombe & English, 1996). Although most transgenes apparently do not produce these effects, the progressive silencing with increasing dosage of the transgene, as, for example, found in *Drosophila*, reflects a biological process referred as a defence mechanism against too many copies of a certain gene (Pal-Bhadra *et al.* 1997).

Effects of the genetic background

Transgenes integrated into an established genome have to develop their function in concert with the many other genes of the cell. Pleiotropic genes and polygenic traits can describe the network of interaction between genotype and phenotype. Phenotypic variations of transgenic lines after changing the genetic background are known. These background effects were, however, only rarely classified and systematically investigated. The influence of background genes, also called modifiers, can heavily influence the characteristics of a transgenic line (Pircher *et al.* 1989; Baribault *et al.* 1994; Hsiao *et al.* 1995; Rozmahel *et al.* 1996; Nagasawa *et al.* 1996; Bonyadi *et al.* 1997). These modifier genes can only be revealed by breeding mutations (a certain transgenic locus) onto different genetic backgrounds. To achieve this, the backcross of the transgene into defined inbred strains is very helpful. This backcross approach is primarily used to establish congenic lines. Marker-assisted breeding (speed congenic) can reduce the time needed for congenic-strain production (Markel *et al.* 1997).

In an undefined genetic background, the polymorphism of modifier genes may be a source of variability that could exacerbate the interpretation of experimental results (Gerlai, 1996; Wolfer *et al.* 1997). On the other hand, the recognition of a modifier gene can be an interesting reference for a better understanding of an observed phenotype or can improve a particular mouse model.

Strategies for site-specific and single-copy transgene integration

The possibility of a directed transgene integration into a neutral chromosomal site would make the pronuclear injection results more reproducible and comparable. Position effects on transgene expression caused by random integration sites thus could be eliminated and could be combined with the ability to avoid undesired insertional mutations.

To achieve a directed transgene integration, the two site-specific recombination systems of *Cre–loxP* and of *FLP–FRT* were thought to be helpful. The simplicity of these experimental systems has led to their widespread use as a tool for *in vivo* DNA recombination. For example, Cre-recombinase was successfully used to delete *loxP*-flanked fragments of chromosomal integrated transgenes (Lakso *et al.* 1992). Although recombination is a reversible process, the integration (intermolecular recombination) is about two orders of magnitude less efficient than the deletion (intramolecular recombination) (Abremski *et al.* 1983). After site-specific recombination, the integrated DNA fragment is flanked by two *loxP* sites and therefore promptly excised as long as the Cre-recombinase is present.

Several strategies were tried to generate a stable insertion product. Most promising was the use of mutated *loxP* sites combined with a transient provision of Cre-recombinase to curtail the post-integration recombinase activity (Senecoff, 1988; Fukushige & Sauer, 1992; Baubonis & Sauer, 1993; Albert *et al.* 1995). Because of very low efficiency this strategy has proved so far to be inapplicable even when it is combined with pronuclear injection (T. Rülicke, unpublished results). Araki *et al.* (1997) succeeded in producing a Cre-mediated single copy integration of transgenes into a mutated chromosomal *loxP* site in murine ES cells. However, the recombination frequency obtained was only comparable to that of gene targeting via homologous recombination. A similar approach by using mutated *FRT* sites in *E. coli* reduced the recombination efficiency by a factor of ~100 compared with the wild-type (Huang *et al.* 1991). Thus, the enhanced stability of integration by the use of mutated *loxP* or FRT sites is probably outweighed by a very inefficient forward recombination.

Targeting a single copy of a transgene to a specific location in the mouse genome can be performed with the help of homologous recombination in ES cells. The transgenic sequence is transferred as part of a targeting construct to the target endogenous sequence. Particularly useful for this strategy is the mutated *Hprt* locus: homologous recombination events are directly selectable and the ubiquitously expressed locus provides an optimal chromosomal environment for transgene expression (Bronson *et al.* 1996). The targeting frequency seems to be insensitive to the length of non-homologous DNA in the vector, rendering this strategy absolutely available for the site-directed integration of classical transgenic constructs (Mansour *et al.* 1990).

Assessing transgenic animals

There is continuous and remarkable development in transgenic technology, particularly in the quality of transgenes and experimental approaches. Nevertheless, the consequences of experimentally induced mutations cannot be completely predicted. This is especially true for the majority of transgenic animals that are generated by pronuclear injection. Even

though our experience shows that the welfare of the majority of transgenic animals is not noticeably affected, randomly integrated foreign DNA may increase the risk of disturbing the normal physiology of an animal. The resulting phenotypic changes may be crucial to the animals' welfare already during breeding and maintenance of a transgenic line. Apparent and relevant phenotypic changes can only be determined after a careful and comprehensive assessment (Mertens & Rülicke, 1999, 2000). This monitoring for phenotypic changes should be done as early as possible, namely during the establishment of a new transgenic line. Since each transgenic line produced by pronuclear injection is unique, it has to be carefully assessed separately by specialists.

Outlook

Pronuclear DNA injection has enabled the scientific community world wide to selectively add defined genes of choice into the germ line of laboratory as well as farm animals. Many experiments with transgenic animals confirmed that transgenesis can provide new insight into many aspects of mammalian life, development and diseases.

Moreover, improved strategies allow the modification and tightly controlled regulation of transgenes after chromosomal integration. Most impressively, the conditional or inducible activities of transgenes can be controlled in a specific spatiotemporally regulated manner and provide great advance in transgenic animal technology.

The use of conditional and inducible transgenic systems can permit the study of a phenotype containing or lacking the transgene product at any given developmental stage of the same individual. This could enable experimental approaches that can either focus on specific time points or in specific tissues during ontogeny, on effects of the duration of transgene expression and on the reversibility of induced phenotypes. These strategies can take advantage, for example, of prokaryotic recombination and transcriptional activation systems such as *Cre-loxP* and *tetR/tetO* (Hoess & Abremski, 1985; Gossen & Bujard, 1992). Recently, the Cre-system has been developed as a standard genetic tool. The expression of Cre-recombinase, for example, can be restricted to a given cell type by the use of a tissue-specific promoter to dictate specific transgene expression or suppression (For details see the Cre-transgenic database: www.mshri.on.nagy/Cre-pub.html). Other strategies to utilize steroid receptors and their associated proteins are being tested in mice. To accomplish tissue- and, additionally, time-specific transgene regulation, the Cre enzyme can be expressed as a fusion protein with a mutant estrogen receptor ligand-binding domain which is exclusively responsive to the synthetic oestrogen antagonist tamoxifen (Schwenk *et al.* 1998).

The tetracycline-regulated system is not yet fully developed to guarantee success in each case. Some of the difficulties may relate to variations between different cell types in their interaction with tetracycline, as found in *in vitro* studies (Ackland-Berglund & Leib, 1995). Moreover, mammalian cells could be an inappropriate environment for processing the bacterial *tetR* gene. It remains to be seen whether this and other systems that are currently under investigation will be more robust for application in transgenic animals.

Controlled simultaneous and/or independent expression of one or several transgenes simultaneously and independently in the same organism is a powerful tool to study the gene function and its physiological consequences. Novel methods and systems for the creation of transgenic animal models shows considerable promise and will broaden their scope of application. As discussed in this review, it is also necessary to bear in mind that highly sophisticated transgenic strategies that are based on pronuclear injection can be subjected to many possible influences.

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Brain Path ology 9: 733-739(1999)

SYMPOSIUM: Tau and Synuclein in Neuropathology

Transgenic Models of Tauopathies and Synucleinopathies

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Rapidly emerging concepts about the pathobiology and defining phenotypes of two major classes of neurodegenerative disease known as tauopathies and synucleinopathies are bringing these diseases into shaper focus. Significantly, recent research has substantially advanced understanding of these neurodegenerative disorders thereby providing fresh opportunities for the development of transgenic (TG) mouse models. Since the availability of such animal models will accelerate efforts to discover more effective therapies, we review the current status of efforts to generate informative TG mouse models for tauopathies and synucleinopathies and other neurodegenerative disorders characterized by prominent filamentous brain lesions.

Introduction

Filamentous brain lesions are hallmarks of many diverse neurodegenerative diseases (see Table 1), most of which are poorly understood and nearly all of which lack effective therapies (for recent reviews, see ref. 7, 8, 12, 15, 17). For example, filamentous tau lesions are characteristic of a group of common as well as rare neurodegenerative disorders referred to as tauopathies (see Table 2), while filamentous α-synuclein lesions are signature brain lesions of another group of diverse and variably frequent neurodegenerative diseases known as synucleinopathies (see Table 3). Indeed, increasing evidence suggests that filamentous aggregates resulting from abnormal protein-protein interactions play a mechanistic role in the dysfunction and death of neurons and/or glia in many neurodegenerative diseases (reviewed in 7, 8, 12, 15, 17). Despite the fact that different proteins form the abnormal filaments in diverse neurodegenerative diseases, it is plausible that similar pathologic mechanisms may cause filament formation. Notably, most of the protein subunits of these abnormal filaments are soluble polypeptides that do not self-assemble into polymeric fibrils in the normal human brain. Further, lesions specific to each of these disorders may share similar toxic properties that compromise the function or viability of affected brain cells. Thus, insights into any one of the diseases listed in Tables 1-3 could provide insights into one or more of the other disorders as well.

The elucidation of these and other hypothetical mechanisms leading to the neurodegenerative diseases will require animal models that recapitulate key phenotypic aspects of these disorders. Recent dramatic advances in understanding tauopathies and synucleinopathies are the focus of the reports in this symposium. Significantly, many of the more recent advances provide new opportunities for the development of transgenic (TG) mouse models of these diseases (7, 8, 12, 15, 17). The availability of animal models would substantially accelerate efforts to discover more effective therapies for tauopathies and synucleinopathies. Therefore, we review the current status of TG mouse models of these diseases and highlight some of the more promising possibilities for the generation of informative animal models for tau and synuclein neurodegenerative brain pathologies in the near future.

FTDP-17 as a prototype for the tauopathies

Filamentous tau inclusions, in addition to extensive neuron loss and gliosis, are hallmark neuropathologic lesions of neurodegenerative tauopathi s including AD, Down's syndrome (DS), several variants of prion dis-

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Disease	Lesion/components	Location
Alzheimer's disease (AD)	SPs/Aβ NFTs/PHFtau	Extracellular Intracytoplasmic
Tauopathies	NFTs/AD-like PHFtau	Intracytoplasmic
Parkinson's disease	LBs/α-synuclein	Intracytoplasmic
Dementia with LBs	LBs/α-synuclein	Intracytoplasmic
LB variant of AD	SPs/Aβ NFTs/PHFtau LBs/α-synuclein	Extracellular Intracytoplasmic Intracytoplasmic
Multiple system atrophy	GCIs/α-synuclein	Intracytoplasmic
Prion diseases	Amyloid plaques/Prions	Extracellular
Amyotrophic lateral sclerosis	Spheroids/NF subunits SOD1	Intracytoplasmic
Tri-nucleotide repeat diseases	Inclusions/Expanded polyglutamine tracts	Intranuclear and dendritic
Neuronal intranuclear inclusion disease	Inclusions/Expanded polyglutamine tracts	Intranuclear

Table 1. Sporadic and hereditary neurodegenerative diseases characterized by prominent filamentous brain lesions. See text for abbreviations.

Neurodegenerative Diseases with Filamentous Tau Lesions
Sporadic/Familial Alzheimer's disease
Amyotrophic lateral sclerosis/parkinsonism dementia complex
Argyrophilic grain dementia
Corticobasal degeneration
Dementia pugilistica
Diffuse neurofibrillary tangles with calcification
Down syndrome
Frontotemporal dementia with Parkinsonism linked to chromosome 17
Gerstmann-Straussler-Scheinker disease
Hallervorden-Spatz disease
Inclusion body myositis
Jakob-Creutzfeldt disease
Multiple system atrophy
Niemann-Pick disease type C
Pick's disease
Prion protein cerebral amyloid angiopathy
Progressive supranuclear palsy
Subacute sclerosing panencephalitis
Tangle-predominant Alzheimer's disease

Table 2. Sporadic and hereditary neurodegenerative diseases characterized by filamentous tau lesions.

Neurodegenerative Diseases with Filamentous Synuclein Lesions
Sporadic/Familial Parkinson's disease
Sporadic/Familial Alzheimer's disease
Dementia with Lewy bodies
Multiple system atrophy
Down syndrome
Hallervorden-Spatz disease
Prion diseases

Table 3. Sporadic and hereditary neurodegenerative diseases characterized by filamentous synuclein lesions.

eases, progressive supranuclear palsy (PSP), amyotrophic lateral sclerosis/parkinsonism-dementia complex of Guam (ALS/PDC), Pick's disease (PiD), corticobasal degeneration (CBD), sporadic frontotemporal dementias (FTDs), hereditary frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) syndromes. (Table 2) Although the tau pathologies in the brains of patients with AD, DS and several other tauopathies coexist with additional diagnostic brain lesions (e.g., abundant deposits of Aβ in AD and DS), the brains of patients with a number of neurodegenerative tauopathies are characterized almost exclusively by prominent tau inclusions. Examples of the latter are PiD, CBD, PSP, ALS/PDC and FTDP-17 (8, 12, 17). Given the location of the tau gene near the linkage site, tau had been speculated for some time to be a candidate gene for FTDP-17; however, there had been little or no evidence to substantiate this hypothesis until Poorkaj and coworkers reported the first pathogenic tau gene mutation (22). This was followed shortly thereafter by other publications (3, 5, 14, 24) leading to the identification of more than 10 different pathogenic tau gene mutations in more than 20 kindreds. Moreover, reports of new tau mutations and the identification of new FTDP-17 kindreds continue to appear, and it is highly likely that additional tau mutations will be discovered as increasing research attention is focused on hereditary FTDP-17 (12, 17). Although FTDP-17 is a genetically determined counterpart of seemingly sporadic FTDs, it must be acknowledged that the role of genetics in this group of FTDs remains largely unexplored. Not all of these disorders are linked to chromosome 17 and not all those linked to chromosome 17 have been shown to have tau mutations. This notwithstanding, the remarkable discovery of pathogenic tau mutations provides unequivocal support for the hypothesis that defects in the tau gene alone are sufficient to cause a neurodegenerative disease.

The discovery of tau gene mutations in FTDP-17 kindreds represents important and unique new opportunities for research into the pathogenic mechanisms of FTDP-17 and related tauopathies. These discoveries also will enable rapid development of animal models of these disorders since FTDP-17 syndromes are autosomal dominantly inherited disorders with diverse clinical features that are characterized by abundant insoluble intracellular filamentous tau inclusions (12, 17). Tau inclusions are abundant not only in neurons, but also in astrocytes and oligodendrocytes in the brains of affected members of some of the FTDP-17 pedigrees (12, 17). Additionally, clinical and pathological features of FTDP-17 syndromes overlap with those seen in several other tauopathies including PSP, CBD, PiD and AD. This may be explained in part by studies that appear to link different intronic and exonic mutations in the tau gene to specific losses of normal tau function or to toxic gains of function. For example, depending upon the topography of a given mutation in the tau gene, a mutation may alter the splicing of tau mRNAs and the finely regulated expression of each of the 6 tau isoforms in the adult human brain; impair the ability of tau to bind to and promote the assembly of microtubules (MTs); or reduce the solubility of tau isoforms and augment their fibrillogenesis (3, 4, 10, 12-14, 17, 24, 27).

Insights into how these tau gene mutations cause brain degeneration benefited from extensive prior understanding of the normal biology of tau proteins that had accumulated as a result of decades of basic research on MTs and MT associated proteins. The role of alterantive splicing and phosphorylation in the generation of various tau isoforms is reviewed by Buée and Delacourte in this symposium. In brief, 6 tau isoforms (i.e. the 3R0N, 3R1N, 3R2N, 4R0N, 4R1N and 4R2N tau isoforms; see also below) are generated by alternative mRNA splicing of 11 exons in the tau gene. Alternative splicing of E10 gives rise to tau isoforms with 3 or 4 MT binding repeats (i.e. 3R and 4R). Further, demonstration that the ratio of 3R tau to 4R tau isoforms in the normal adult human brain is ~1 suggests that the alternative splicing of the tau gene is tightly regulated (13).

Intronic and exonic tau gene mutations that affect E10 splicing as well as exonic mutations leading to missense substitutions at or near the MT binding repeats that impair tau functions (including the ability of tau to bind to and promote MT assembly) have been identified in families with FTDP-17 (3,4,10,12-14,17,27). Further, these and other studies indicate that multiple mechanisms are operative in regulating E10 splicing. Increased E10 splicing augments expression of 4R tau proteins in the brains of FTDP-17 patients. In addition to E10 splice mutations, several tau missense mutations located in or near MT-binding repeats appear to cause FTDP-17 by altering the biochemical properties of tau as well as the functional interactions of tau with MTs. Thus, emerging data suggest the hypothesis that the topography of each tau gene mutation (i.e. FTDP-17 tau genotype) is critical to disease pathogenesis and predictive of a specific tau dysfunction (3, 4, 8, 10, 12-14, 17, 24, 27).

The discovery of E10 splice mutations in a subset of FTDP-17 kindreds has provided important clues for developing a better understanding of seemingly sporadic tauopathies including PiD, CBD and PSP. Thus, the selective aggregation of insoluble 3R tau or 4R tau isoforms as cytoplasmic inclusions in familial and sporadic neurodegenerative disorders suggests that the de-regulation of E10 splicing may be a fundamental pathogenic mechanism in the tauopathies. Since it is plausible that different forms of familial and sporadic tauopathies can be caused by perturbing any of the multiple and complex mechanisms that regulate the stability, splicing and functions of the tau gene and the proteins it encodes, further analyses of tau gene regulation and tau protein expression will undoubtedly lead to new approaches to understanding the pathogenesis of tauopathies.

Although the notion that tau might play a fundamental role in the onset and progression of neurodegenerative diseases has been regarded with skepticism since the discovery of familial AD (FAD) mutations, the discovery of pathogenic tau mutations directly demonstrates that tau abnormalities can cause neurodegeneration in the absence of Aβ deposits. For example, it is possible that some of the missense FTDP-17 tau gene mutations might cause disease by impairing the ability of tau to bind MTs which could destabilize MTs, disrupt axonal transport, lead to dying back of axons and the death of neurons. Alternatively, the formation of intracytoplasmic filamentous tau inclusions could represent a gain of toxic function leading to the death of affected cells, and these inclusions could be due to alterations in ratio of 4R tau to 3R tau isoforms that are the consequence of other FTDP-17 mutations in tau introns. Indeed, although the formation of tau pathologies may be downstream consequences of FAD mutations, the development of these pathologies could be an essential and necessary mechanistic step for the degeneration of neurons in FAD. Accordingly, tau dysfunction and filamentous tau aggregates must be considered plausible targets for the development of novel and more effective drugs for the treatment not only of FTDP-17, but also for the treatment of AD. Moreover, such therapies also

may be relevant t the treatment of other tauopathies since tau pathologies might initiate neurodegenerative disease or represent a final common pathway in the relentlessly progressive brain degeneration associated with many of these brain disorders (12,17). For these reasons, TG mouse models of FTDP-17 tauopathies also may serve as informative models for research into the role tau pathologies play in the onset and progression of AD and related tauopathies.

Tau transgenic mice

Although TG mouse lines over expressing 3R0N or 4R2N or human tau using cDNAs driven by 3-hydroxy-3-methylglutaryl coenzyme A reductase and Thy-1 promoters, respectively, resulted in "pre-tangle" tau pathology, but no filamentous tau inclusions (2, 9), it must be emphasized that efforts to produce animal models of tau pathologies have been very limited (8, 12, 17). In contrast, many years of intense, but failed, efforts to generate TG mice with Aβ deposits by investigators in a large number of laboratories preceded the eventual achievement of a TG mouse model of AD amyloidosis (6). Thus, intense empirical efforts to develop TG mouse models of tau pathologies are needed and justifiable now, and these efforts should be greatly facilitated by the discovery of the FTDP-17 tau gene mutations. It may be necessary to express transgene-derived tau proteins at higher levels than those achieved in the previous lines of tau TG mice to induce filamentous tau inclusions.

Notably, intraneuronal injections in the lamprey of tau cDNA containing plasmids followed by the massive over expression of 4R2N human tau has been shown to lead to the formation of inclusions formed by aggregated tau fibrils that appeared similar to paired helical filaments (PHFs) in AD NFTs. This was associated with the degeneration of some affected neurons in the lamprey central nervous system (11). Encouraged by these results, and the formation of "pre-tangle" tau pathology in the TG mice described earlier (2, 9), we recently produced TG mouse lines that express 5-10 fold higher levels of wild type human 3R0N tau than endogenous mouse tau. These mice developed filamentous intraneuronal inclusions composed of tau and neurofilaments (NFs), but the fibrils in these inclusions exhibited the ultrastructural features of straight filaments rather than PHFs characteristic of AD NFTs (unpublished observations). With advancing age, these TG mice acquired a phenotype that was more similar to some variants of FTDP-17, PSP and ALS/PDC rather than to AD. Although some NF proteins do occur in variable numbers of NFTs, NF proteins appear to accumulate after tau during the formation of NFTs (23). Accordingly, it will be important and informative to cross these tau TG mice with NF knockout mice to attempt to develop filamentous tau tangles without NFs.

In future models it may prove useful to utilize constructs with tau gene mutations, including those mutations that are considered to act by either a loss of tau function or a toxic gain of function, to generate tau TG mice. In addition to neuron specific promoters, it also will be critical to generate TG mice using glial specific promoters to drive transgene protein expression. The reason for this is that tangles occur in glia in many tauopathies (12, 17), and the development of TG mouse models that acquire glial tau pathology will enable investigation of disease mechanisms that lead to the dysfunction of affected glial cells. Finally, minigenes that enable overexpression of all 6 human tau isoforms with and without intronic or exonic FTDP-17 tau gene mutations using mouse tau gene knock-out animals may lead to more authentic TG mouse models of tauopathies. Crossing these TG mice with TG mouse models of AD amyloidosis may result in model systems that more accurately recapitulate the tangle and plaque pathology of AD. Thus, using model systems like those discussed here, it should be possible to make rapid progress in elucidating how and why glial and neuronal tau pathologies lead to the onset and progression of diverse sporadic and hereditary tauopathies in the very near future.

Synucleinopathies

A few years after a peptide derived from α-synuclein (i.e. "NAC" or the non-amyloid component of amyloid) initially implicated this synaptic protein in AD (7, 15), the A53T and A30P α-synuclein gene mutations were discovered in rare familial PD kindreds (16, 21). This was rapidly followed by reports showing that α-synuclein is a major component of Lewy bodies (LBs) and Lewy neurites in sporadic Parkinson's disease (PD), dementia with LBs and the LB variant of AD. Antibodies to α-synuclein were shown to detect more Lewy neurites and LBs than previous antibodies. Both normal and mutant α-synuclein were demonstrated to assemble into filaments like those seen in LBs (1, 7, 15, 25). Thus, these discoveries have re-focused attention on the increasingly compelling hypothesis that LBs and Lewy neurites play a mechanistic role in the degeneration of affected neurons in Lewy body disease (7,15). Moreover, mutations in the presenilin and Aβ precursor protein genes have been linked to accumulations of many α-synuclein positive LBs in >60% of FAD brains,

and in >50% of DS brains with AD path logy. More recently, α-synuclein has been shown to form filamentous glial cell inclusions (GCIs) in oligodendroglia of multiple system atrophy (MSA) (7, 15). In addition, it is well known that AD and PD occur in the same patient more frequently than expected, and although the mechanisms that account for this have not been clarified (20), it is plausible that α-synuclein plays a role in these mechanisms. Taken together, there now is compelling support for the hypothesis that filamentous α-synuclein inclusions play a mechanistic role in the pathogenesis of neurodegenerative disorders (7, 15).

Further support for this hypothesis comes from studies showing that TG mice that develop LB-like NF inclusions show evidence that the viability of affected neurons is compromised with advancing age, and they are more vulnerable to degenerate following traumatic brain injury (19, 26). Moreover, LBs and dystrophic Lewy neurites could be deleterious even to those neurons that harbor these lesions and survive for a period of time. If α-synuclein lesions disrupt transport of organelles and proteins in perikarya and processes of affected neurons, this may be followed by a "dying back" of processes and disconnection of neuronal circuits.

Synuclein transgenic mice

Although no full-length reports have been published describing failed or successful efforts to produce TG mouse models of synucleinopathies by over expressing normal or mutant human α-synuclein, it is likely that such reports will appear soon. Indeed, at least one α-synuclein TG mouse line has been generated, and it was reported to develop perikaryal α-synuclein aggregates, but it remains unclear if these aggregates are associated with neuronal dysfunction or degeneration (18). Efforts to generate α-synuclein TG mice with a neurodegenerative phenotype will face a number of hurdles. For example, it will be necessary to design TG mice that take into account the fact that the normal mouse a-synuclein protein harbors the A53T substitution as well as the fact that there are a number of other synucleins (e.g., β-synuclein, γ-synuclein, synoretin) that are products of distinctly different genes, but also are expressed in the brain (7, 15). Thus, it may be necessary to generate TG mouse models of synucleinopathies using a strategy involving α-synuclein, β-synuclein, γ-synuclein and synoretin knockout mice (like the NF knockout mice described above for tauopathy TG mouse models) so that normal and mutant human α-synuclein can be overexpressed in TG mice without the potential confounds of endogenously expressed mouse synucleins. Moreover, crosses of such mice with ther TG mouse models of AD amyloidosis and tauopathies offer the prospect of developing animal models of LB variant of AD and forms of FAD and DS with associated LB pathology. While this may appear to be a daunting task, the aggregation of brain proteins is emerging as a common mechanistic theme in several sporadic and hereditary neurodegenerative diseases and the remarkable advances in understanding the pathobiology of PD and related synucleinopathies will stimulate intense efforts to develop TG mouse models of these and related disorders in the near future,

Summary

Current understanding of the mechanisms that convert normal soluble tau and α-synuclein into insoluble filamentous aggregates characteristic of tauopathies and synucleinopathies, respectively, is still very fragmentary, and advances is this area of research have been impeded by the lack of animal and cell culture models. Although PHF-like tau filaments as well as LB-like and GCI-like α-synuclein filaments can be produced in a test tube, the conditions required are highly artificial, and *in vitro* paradigms have limited utility as models of *in vivo* mechanisms of neurodegeneration. Thus, it is essential to develop TG mouse models of these filamentous lesions, despite the fact that previously published efforts to produce TG models of tauopathies did not result in TG mice that acquired filamentous tau lesions (2, 8, 9). However, the injection of constructs into lamprey neurons to overexpress tau did result in somatodendritic tau expression, PHF-like filament formation as well as evidence of nerve cell degeneration in this simple vertebrate (11). Thus, these studies taken together with our own unpublished data on tau TG mice provide proof of the concept that, when sufficiently overexpressed in neurons, tau can be induced to form filaments that result in neuron degeneration. Moreover, the recent identification of tau gene mutations in FTDP-17 syndromes, and advances in understanding the pathobiology of other tauopathies as well as the role of α-synuclein in PD and related synucleinopathies open up fresh opportunities to develop TG mouse models of these neurodegenerative diseases in the near future. Significantly, while these TG mouse models will be critical for studies to address important questions about mechanisms of disease in tauopathies and synucleinopathies, they also may help clarify mechanisms of brain degeneration in a larger group of other neurodegenerative diseases characterized by filamentous brain pathologies. Finally and most importantly, these models also can be exploited for test-

ing potentially effective therapeutic agents targeted at mechanisms of brain degeneration in tauopathies, synucleinopathies and other neurodegenerative disorders.

Acknowledgements

The authors thank members of their laboratory and their collaborators within and outside the University of Pennsylvania for their important contributions to the studies reviewed here. The families of the many patients studied by our group over the past decade have made it possible to pursue many of the research advances discussed here. The studies summarized here from our laboratory were supported by grants from the National Institute on Aging of the National Institutes of Health, the Dana Foundation and the Alzheimer's Association.

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TRANSGENIC LIVESTOCK: PROGRESS AND PROSPECTS FOR THE FUTURE

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ABSTRACT

The notion of directly introducing new genes or otherwise directly manipulating the genotype of an animal is conceptually straightforward and appealing because of the speed and precision with which phenotypic changes could be made. Thus, it is of little wonder that the imagination of many an animal scientist has been captivated by the success others have achieved by introducing foreign genes into mice. The private sector has embraced transgenic livestock technology resulting in the formation of two new industries. However, before transgenic farm animals become a common component of the livestock production industry, a number of formidable hurdles must be overcome. In this brief communication, the technical challenges are enumerated and possible solutions are discussed.

Key words: transgenic livestock, gene transfer, microinjection

INTRODUCTION

The definition of transgenic animals is evolving. For the purpose of this paper a transgenic animal is one containing recombinant DNA molecules in its genome that were introduced by intentional human intervention. In this review I will focus on animals in which transgenes were introduced into preimplantation embryos by pronuclear microinjection, with the intended consequence of producing germline transgenics as opposed to somatic cell transgenics. Though there are other means of introducing genes into preimplantation embryos (20,29), pronuclear microinjection, basically as originally described by Jon Gordon (25), and as modified for livestock in our laboratory (65), is still the predominant method employed.

Acknowledgments

Many of the concepts, conclusions and visions of the future included in this manuscript have evolved over the years from discussions at our Friday afternoon lab meeting. Vern Pursel and Caird Rexroad, Jr., who pioneered transgenic livestock technology, provided the leadership. In recent years Ken Bondioli, David Kerr, Paul Hyman and Uli Tillmann have provided valuable new insights and new approaches that have and will advance the field.

Theriogenology 45:57-68, 1996

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0093-691X/96/$15.00
SSDI 0093-691X(95)00355-X

WHY MAKE TRANSGENIC ANIMALS?

A Medline search reveals that over 6,000 scientific articles have been published in which transgenic animals (mostly mice) were used to answer basic research questions. By contrast 289 papers dealt with transgenic livestock, of which 24% were reviews. The limited publication record for transgenic livestock species reflects the high costs and technical difficulties associated with producing transgenic livestock more than lack of applicability of this technology to farm animals. A number of well defined goals have been enumerated in the numerous review articles written by animal scientists. Not surprisingly, many of the proposed applications closely parallel the long term objectives of animal agriculture.

In theory, transgenic technology provides a mechanism by which economically important traits can be attained more rapidly than by selective breeding without concern of propagating associated, possibly undesirable, genetic characteristics. If genetic precision and speed of improvement were the only advantages of transgenic technology, use of that methodology might be difficult to justify. That is because current cost of producing transgenic animals are high and understanding of the appropriate genetic manipulations required to influence economically important traits is limited. However, transgenic technology offers much more. Genes can be transferred across species boundaries and can be modified to function very differently than they do in their native form (gene products, tissue specificity, and timing of expression can be altered). The ability to redirect expression of genes to another organ has spawned the transgenic bioreactor industry. For the most part, transgenic bioreactors are farm animals designed to produce new proteins in their milk or other body fluids. It is envisioned that this approach will have application in both food production and the biomedical arena. Modifying the composition of milk through genetic engineering is the topic of Dr. Bremel's paper in these proceedings and will not be dealt with here.

TRANSGENIC LIVESTOCK PROJECTS

For the sake of brevity, only a very brief summary of the 37 gene constructs that have been tested in livestock will be reported here. The reader is referred to two excellent reviews that list those constructs and their consequences (16,53).

The Transgene.

The power of transgenic technology is derived from the introduction of genetic information with new functionality. The strategy for building a transgene (fusion gene) involves selecting a genetic regulatory element (often called promoters, but usually containing both an enhancer element and a promoter) that will determine the tissue in which the gene is to be expressed and the time and magnitude of expression. In some cases, the regulatory element can act as a switch, allowing the transgene to be turned on and off at will. The second part of the gene construct consists of DNA sequence encoding the desired protein (often referred to

Theriogenology

as the structural component of the transgene). For example, in the first transgenic livestock experiment (28) we wanted to increase the levels of circulating growth hormone in a controlled manner. The gene construct used to accomplish this consisted of the regulatory element of a metallothionein (MT) gene fused to the coding sequence for growth hormone (GH). Metallothionein is an inducible liver enzyme, and its gene is usually quiescent (turned off) until a threshold level of circulating zinc or cadmium triggers transcription. Therefore, it was expected that the MT-GH fusion gene would be silent until the animals were fed zinc. In those experiments GH expression could be inducted but, in most cases, the transgene could not be turned off completely. New more complex inducible approaches are now being tested (23,26). These new systems rely on tetracycline or its analogs to activate or repress transgene expression. It is too early to know if these strategies, in their current form, will be more tightly regulated then the MT system. However, if they are not, the general paradigm on which the new systems are based will probably lead to improved inducible systems.

Applied Transgenic Projects.

The vast majority of original research reports have focused on growth enhancement. Growth hormone (GH) was the structural gene employed in 13 of those publications and the gene for growth hormone releasing factor in four. Other structural genes tested include IGF-1, cSKI and an estrogen receptor. The regulatory elements derived from MT genes, from various species, were most frequently used, appearing in nine of the growth-related fusion genes. Long terminal repeats (LTR) from two retroviruses, MLV and RSV, and sequence from CMV, a DNA virus, served as regulatory components of transgenes, as have the promoters from albumin, prolactin, skeletal actin, transferrin and phosphoenolpyruvate carboxykinase (PEPCK) genes. All but two of 21 growth constructs were tested in pigs and the most striking phenotypes resulted from the use of MT-GH fusion genes (53).

Seven transgenes designed to enhance disease resistance and to produce immunologically-related molecules have been introduced into pigs and sheep (5,13,41,67). Though desirable expression patterns have been reported in several of the projects, none of the studies has progressed to the point of demonstrating a beneficial effect of transgene products.

Very recently it has been reported that transgenic sheep with enhanced wool production characteristics have been produced (9). The results are quite promising; if no unforeseen anomalies occur, transgenically produced wool maybe the first marketed livestock product.

Biomedical Transgenic Projects.

Other proposed transgenic farm animal applications are decidedly non-agricultural in nature. One of the first transgenic animal companies demonstrated the feasibility of producing new animal products by manufacturing human

hemoglobin in pigs, to serve as a principal component of a human blood substitute (59). Human antibodies have also been produced in transgenic mice (62). Another area where transgenic animals, especially pigs, will have a significant impact on society will be in the development of human genetic disease models. To date, genetic disease models have been generated in mice for atherosclerosis (6), sickle cell anemia (18), Alzheimer's disease (21), autoimmune diseases (44), lymphopoiesis (33), dermititis (55), and prostate cancer (61). These models for the most part require "knocking out" the function of a gene or replacing an existing gene with a mutant form. Many of these models will have to be replicated in farm animals to be useful. Unfortunately, the stem cell technology required to generate most of the disease models is still in development for livestock (51).

Finally, a new use not reported in the above mentioned reviews deserves note. The objective of this new endeavor is to genetically engineer animals, primarily pigs, so that their organs can be used as xenografts for humans. Preliminary studies to test the concept have been performed in mice (40,42) and transgenic pigs have now been produced (19,54). Though several strategies are being explored, the general approach has been to block activation of complement, which is normally part of the acute transplantation rejection response. These organs are intended for temporary use, until an appropriate human organ becomes available. However, as the technology develops, a driving force will be the design of transgenic organs for extended use or permanent transplantation.

CHARACTERISTICS OF TRANSGENIC ANIMALS

Transgenic livestock projects are costly, primarily because the process is inefficient. Production costs range from $25,000 for a single founder pig to over $500,000 for a single functional founder calf (64). The calculation for cattle was based on obtaining zygotes by superovulation of embryo donors, the normal practice for all mammalian species. However, the costs are reduced by as much as a third if oocytes derived from ovaries collected at slaughter are the starting material. The remainder of this review will be devoted to characterizing the transgenic animal model, to identify points in the process that reduce efficiency, and finally discussing possible approaches that have been proposed to overcome major hurdles to progress.

Transgene Integration.

Even though several hundred copies of a transgene are microinjected, any transgene that becomes incorporated into the genome generally does so at a single location. Exceptions are rare (58). Thus, transgenic founder animals are hemizygous for transgenes. It is also common for a transgene locus to contain multiple copies of the transgene, arranged in a head-to-tail array. These two characteristics of transgene loci should provide clues to the mechanism by which transgenes integrate. So far, few researchers have formulated compelling hypotheses to explain the event (2,47) and the hypotheses that have been proposed remain untested.

Without knowledg
to devise approache

Transgene i
animals (cattle, sh
and rats, Table 1).

Table 1. Example
several laboratorie

Species	Injected & transferr d embryos (No.)
Mice	12,314
Rabbits	1,907
Rat	1,403
Cattle[c]	1,018
Pigs	19,397
Sheep	5,424

[a] Number of experime
tested.
[b] The value for cattle i
[c] Eleven thousand tw
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Without knowledge of the molecular mechanism it is going to be extremely difficult to devise approaches to make transgene integration more efficient.

Transgene integration efficiency is low and ranges from about 1% in farm animals (cattle, sheep and pigs) to about 3% in laboratory animals (mice, rabbits and rats, Table 1).

Table 1. Examples of embryo survival and transgene integration efficiencies from several laboratories.

Species	Injected & transferred embryos (No.)	Studies[a] (No.)	Transgenic animals produced: Offspring[b] (No.)	Per Offspring (%)	Per embryo injected & transferred (%)	Refs.
Mice	12,314	18	1847	17.3	2.6	(63)
Rabbits	1,907	1	218	12.8	1.5	(28)
Rat	1,403	5	353	17.6	4.4	(45)
Cattle[c]	1,018	7	193	3.6	0.7	(30)
Pigs	19,397	20	1920	9.2	0.9	(53)
Sheep	5,424	10	556	8.3	0.9	(53)

[a] Number of experiments, which in most cases was equivalent to number of different gene constructs tested.

[b] The value for cattle includes both fetuses and live born calves.

[c] Eleven thousand two hundred and six eggs were microinjected and cultured. One thousand and eighteen developed to morula or blastocysts and were transferred into recipient cows.

Transgene Expression.

Even after the one in 33 to one in 150 injected and transferred eggs results in a transgenic animal the efficiency of the process is further diminished by failure of the transgene to be transcribed. Transgenes are expressed (transcribed) in only about half of transgenic lines, though some specific transgenes are expressed in a higher proportions (15,27). If a founder expresses its transgene, so do its transgenic offspring. It is not clear why some transgenes are expressed in all lines and others in only half the lines. Transgenes are sometimes activated in unintended tissues (ectopic expression), and timing of expression can be shifted relative to development. Our lack of understanding of essential genetic control elements makes it difficult to design transgenes with predictable behavior. The abberent expression patterns (no expression or wrong expression) seen in some linese of transgenic animals has been attributed to the so-called "position effect." If a transgene lands near highly active genes, the transgene's behavior maybe influenced by endogenous genes. Other transgenes may locate in transcriptionally inactive (heterochromatin) regions. The transgene may function normally or be completely silenced by the

heterochromatin. It is likely that both of these factors (position effect and unidentified control elements) contribute to the [illegible] and variable expression in transgenic animals. Some of these problems will be obviated by use of "boundary" DNA sequences that block the influence of surrounding genes (34,43). Refining transgenic technology for farm animals will remain a challenging task in part because experimentation will often have to be conducted in the species of interest. That is because transgene expression and the physiological consequences of transgene products in livestock are not always accurately predicted in transgenic mouse studies (28,48).

Transgene transmission.

Because founder animals are usually single integrant hemizygous for the transgene, one would expect 50% of their offspring to inherit a copy of the transgene locus. This is true for about 70% of transgenic founder mice (49). The remaining founders either do not transmit transgenes to their offspring or transmit transgenes at a low frequency (52,53). It is commonly thought that the non-Mendelian inheritance is the result of transgene mocasicism in germ cells. This could be caused by late integration of transgenes during embryonic development (66). It has been proposed that non-Mendelian inheritance patterns can also be caused by diminished fertilizing ability of transgene bearing sperm (17). The latter explanation may be a special case, because the thymidine kinase gene used in that study was inadvertently expressed in testes.

POTENTIAL SOLUTIONS FOR IMPROVING EFFICIENCY

Testing Transgenes.

Because the "rules" for transgene design are still vague, it is important to have a reliable system for testing gene constructs. The most cost effective method of characterizing the performance of a transgene is cell culture transfection studies. Unfortunately, such studies have a low predictive value (50). The next most cost effective method for testing gene constructs is production of transgenic mice, which as mentioned above do not faithfully predict a transgene's performance in livestock species. Nevertheless, a reasonable amount of useful information about transgene function can be derived from transgenic mouse studies. Currently, the only approach that yields truly informative data is testing transgenes in the livestock species of interest. This is obviously an unsatisfactory, time consuming, expensive testing option. One alternative approach that we are exploring is based on the fact that transgenes will function after being "shot" into somatic tissue. We have been focusing our efforts on the mammary gland, but almost any target organ should be amenable to this approach. We have recently demonstrated that both RNA and protein can be detected following introduction of transgenes into sheep mammary tissue, *in situ* (22,37). Once we confirm that "gene-gunned" transgenes function as they do in transgenic animals, this approach should dramatically reduce the costs and time of evaluating gene constructs.

Improving Integration Frequency

From Table 1 it is clear that integration rates are lower for livestock species than for laboratory animals. Eggs of livestock species are more difficult to microinject than eggs of laboratory animals. However, competent microinjectors can reliably inflate pronuclei with DNA-containing solutions. Furthermore, integration problem occurs after the transgene is deposited. But timing of microinjection may contribute to differences in integration efficiency. It is thought that transgene integration occurs during DNA replication (2), so it would be advantageous to microinject before or during early S-phase preceding the first mitotic division. For the most part that is when laboratory animal eggs are microinjected, but microinjections are apparently performed during late S-phase or later in livestock species (for a full discussion see (63)). Efforts to inject *in vitro* fertilized bovine zygotes early have failed because of difficulties in visualizing pronuclei (K. Bondioli, personal communication and unpublished data). Efforts to synchronize microinjection and S-phase in bovine zygotes have thus far not been fruitful (24).

One way to insure that the transgene is in place before the first mitotic S-phase is to introduce the transgene at fertilization. That could be achieved by sperm-mediated gene transfer (4,38). Notwithstanding the controversy this approach has generated (8), it clearly represents an intriguing method that shows some promise (57). Accumulating evidence suggests that sperm of several species can bind transgenes (11,32,39,68) and carry the genes into oocytes where in some cases the gene persists (4,12,31). However, it appears that in almost all cases, the transgene DNA becomes rearranged or otherwise mutated by the process (Corrado Spadafora, personal communication). Another potential sperm-based delivery approach has been foretold by a pioneering study conducted by Ralph Brinster (7). In that study, transplanted spermatogonial cells generated sperm capable of fertilizing oocytes and offspring were produced. If a means is found to culture, transfect and select spermatagonia with transgenes, Brinster's transplantation scheme could be used to produce transgenic animals. Others have proposed directly transfecting testes as a means of transforming sperm (56).

Retroviral-mediated gene transfer is also a potentially alternative approach for introducing transgenes into embryos with high efficiency (29,36). Though the technique solves the low integration frequency problem, it creates other inefficiencies by generating mosaic founders that may not transmit their transgene. Furthermore, retroviruses can carry only a limited amount of exogenous DNA and therefore the technique limits the size of transgenes. If cDNA based transgenes, which are relatively short, were efficiently expressed, the transgene size restriction would not be a significant problem. However, many cDNA based gene constructs are poorly expressed in transgenic animals (66).

Selection of transgenic embryos.

With no obvious or immediate solution for improving integration frequency, what else can be done to increase efficiency of producing transgenic livestock? One of the most widely discussed approaches is selection of transgenic embryos before they are transferred to recipients (1,14,35,46). If transgenic preimplantation embryos can be identified by analyzing embryo biopsies with the polymerase chain reaction (PCR), the number of recipients required could be greatly reduced. For example in Dr. Bondioli's study ((30), Table 1), 1,018 bovine embryos were transferred into over 1000 cows resulting in seven transgenic calves and fetuses. If embryo selection had been possible, fewer than 20 recipients would have been required. Unfortunately, mounting evidence suggests that this approach will not work. In two very similar studies (10,14) microinjected mouse embryos were cultured to the 8-cell stage, and blastomeres were isolated and analyzed for the transgene by PCR. In our study (10) none of the 8-cell embryos had transgenes in more than 4 blastomeres. We speculate that immediately upon microinjection, transgene copies join to form multi-copy circular arrays. One of these arrays may eventually become integrated, while the non-integrated arrays segregate as daughter blastomeres are formed. If integration occurs after the one-cell stage, some blastomeres may not contain an array, even though the embryo is transgenic. The converse is also possible (all blastomeres acquire arrays but none integrate). Analysis of embryo biopsies could therefore be misleading.

Another scheme for selecting transgenic embryos before transfer is based on expression of a selectable marker-containing transgene. The preliminary results from two recent studies (3,60) appear to be promising. In both studies, transgenes containing a neomycin resistance gene (neo) were microinjected into pronuclei of mice (60) or bovine (3) embryos. The embryos were then cultured in the presence of G418, a neomycin analog, in the hope of killing embryos that did not express the neo gene. Because this approach is based on gene expression and because transgenes can be expressed without being integrated, embryos containing unintegrated copies of the transgene could survive the selection process. However, since G418 interferes with protein synthesis, the blastomeres that expressed the neo gene would have a developmental advantage over those that did not. Therefore, the blastomeres expressing the neo gene might divide more rapidly and have a higher probability of participating in the formation of the inner cell mass (66). Further studies will have to be conducted to determine if this scheme has merit.

In The Future

The tools for gene transfer are in hand, albeit the process is inefficient. Over the next decade, bioreactor and xenograft industries will mature and useful new products will be marketed. The value of possible products will drive the technology as funding for basic research from conventional sources becomes increasingly limited. Researchers will need to develop a better understanding of how mammalian genes are controlled, and identify key genes in regulatory pathways of

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phenotypic characteristics that are to be altered to bring the fruits of this technology to animal agriculture. There is a serious need to transfer transgenic animal technology from a few practitioners to many more laboratories worldwide. Progress in the field will be limited as long as the capabilities to explore this potentially powerful tool is only in the hands of a few. To entice other scientists, the efficiency of producing transgenic farm animals will have to be improved. But the horizon looks bright. Many recently trained animal scientists are now equipped with the knowledge and technical skills needed to advance this technology.

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Perspectives Series: Molecular Medicine in Genetically Engineered Animals

Transgenesis in the Rat and Larger Mammals

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Advances in biotechnology over the last ten years have made it possible for the researcher to alter gene expression in vivo in many diverse ways (1). With the establishment of embryonic stem (ES)[1] cell technology (2), more subtle and precise alterations can now be achieved than were previously possible using microinjection techniques. However, to date germline transmission has only been achieved with mouse ES cells, and microinjection continues to be the method most widely used for other species. While the mouse has a number of advantages, not least the depth of our knowledge of its genetics, other species are being increasingly used for transgenic studies due to their greater suitability for addressing specific questions. We will briefly review the application of transgenic technology to nonmurine species as it stands at present, with particular emphasis on developments appertaining to biomedical research.

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Received for publication 29 January 1996 and accepted 5 February 1996.

1. *Abbreviations used in this paper:* DAF, decay accelerating factor; ES, embryonic stem; HAR, hyperacute rejection; ICM, inner cell mass.

Transgenesis by pronuclear injection

A number of significant limitations regarding the application of pronuclear injection to nonmurine animals have been identified (3), not least being the time and cost. Such limitations are due to longer gestation and generation times, reduced litter sizes, and higher maintenance costs. Further consideration must be given to the large numbers of fertilized eggs (and hence donor animals) required for microinjection, the high cost of carrying nontransgenic offspring to term, and the relatively low efficiency of gene integration. Such limitations are particularly severe for the production of bovine transgenics and, as a consequence, more significant departures from the standard procedures used for the mouse have been adopted for this species (4). For example, the use of in vitro embryo production in combination with gene transfer technology has played a large role in the development of transgenic cattle. The development of microinjected embryos through to the morula/blastocyst stage in recipient rabbits or sheep, enables sexing, transgene screening, and cloning to take place before reintroduction into the natural host, providing that such screening methods are robust and reliable.

The major problem regarding pronuclear microinjection is that the exogenous DNA integrates randomly into chromo-

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0021-9738/96/04/1557/04 $2.00
Volume 97, Number 7, April 1996, 1557–1560

somal DNA. Position effects, where the transgene is influenced by its site of integration in the host chromosome (5), can have major consequences on the expression of the transgene, including loss of cell specificity, inappropriately high copy number–independent expression and complete silencing of the transgene. This is of greater concern in nonmurine transgenesis where the investment is higher. Position-independent, copy number–related expression can be achieved using sequences such as the locus control regions identified upstream of the β-globin gene cluster and downstream of the CD2 gene (6, 7), the A elements which flank the chicken lysozyme gene (8), and matrix attachment regions (9). Such elements have been shown to function across species barriers, and their incorporation into gene constructs can overcome position effects and improve expression of heterologous genes within specific cell types (5). In many cases, simply including large amounts of flanking sequences may be sufficient to overcome position effects and direct expression to specific tissues. To this end, the development and use of P1 (10), bacterial artificial chromosome (BAC) (11) and yeast artificial chromosome (YAC) vectors (12) for cloning of large segments of DNA, should greatly improve the chances of including important regulatory elements, including those involved in chromatin structure, within the transgene construct.

Embryonic stem cell technology

With the development of ES cell technology in the mouse (2), genetic manipulations can be performed in cell culture using appropriate selection strategies to permit the directed integration of the transgene to a specific region of the chromosome via homologous recombination. With the advent of homologous recombination, the researcher is able to insertionally inactivate, replace, or introduce subtle alterations to the endogenous gene of interest. Once the intended genetic change has been verified, the appropriate ES cells are introduced into blastocysts by microinjection, and, during subsequent gestation, may contribute to the developing embryo. If such a contribution is made, then by definition the resulting animal would be chimeric, being derived in part from the ES cells originating in culture. Assuming that the chimerism extends to the germline, then an appropriate breeding strategy will lead to the recovery of nonchimeric heterozygotes and, if viable, mice which are homozygous for the genetic change.

Most attempts to isolate and culture inner cell mass (ICM) cells from other species are based on the methods used for the mouse. ES cells are maintained in culture in the presence of mouse-derived differentiation-inhibiting agents, provided either as a media supplement or through cocultivation in the presence of feeder cells. It has been suggested that these mouse-derived agents do not adequately prevent differentiation of stem cells in species other than the mouse, and pluripotent rat ES cells, capable of producing chimeras, were found to grow best on primary rat embryonic fibroblasts as the feeder layer (13). Freshly isolated cells from ICMs have been injected into blastocysts to produce chimeric offspring in both sheep and cattle (14), and their totipotency at this stage is further demonstrated by their ability to produce offspring after transfer into enucleated oocytes (15). Such nuclear transfer techniques are potentially very useful for the production of clonal offspring and would avoid the initial chimeric generation necessitated by the injection of ES cells into blastocysts. Recently, bovine-specific culture methods have shown promise with cells of up to 27 d of age maintaining their ability to direct normal calf development following nuclear transfer (16). However, at the present time the reliable generation of bovine ES cell lines requires the pooling of ICMs from several blastocysts and further efforts are required to enable the long-term culture of clonal bovine ES cells. Although to date chimeric animals have been generated from several species including the pig (17), in no species other than the mouse has germline transmission of an ES cell been successfully demonstrated. This remains a major goal for the future and may well require the use of novel strategies which depart widely from the traditional methods used in the mouse.

Nonmurine species in biomedical research

Selected physiological questions may be more conveniently modelled in the rat or in larger species. Not only can physical size be an advantage for biochemical sampling and physiological analyses, but certain genes may provide useful information when introduced into, for example, the rat genome when parallel experiments in the mouse would be ineffective. Examples include the modulation of blood pressure by the mouse *Ren-2* gene (18) and the modeling of inflammatory disease (19). In both cases, but for different reasons, no phenotype was observed in the respective transgenic mice, highlighting one of the advantages of having alternative species for understanding physiological mechanisms and the etiology of disease. More recently, a number of transgenic experiments have been undertaken to investigate lipoprotein metabolism. The human apolipoprotein A-1 gene was successfully expressed in the rat (20), resulting in increased serum HDL cholesterol concentrations, and attempts to therapeutically lower apo B100, and hence LDL and lipoprotein(a) concentrations, in the rabbit were successful (21) but resulted in complications. Although the targeted expression of the apo B–editing protein in the liver of the transgenic rabbits resulted in reduced LDL and lipoprotein(a) concentrations as intended, many of the animals developed liver dysplasia, suggesting that high level expression of the editing protein had unforeseen and detrimental side effects, possibly via the editing of other important mRNAs. The rabbit has also been used in HIV-1 research, with the development of a line expressing the human CD4 protein on T lymphocytes (22). Susceptibility to HIV infection was demonstrated, and although the rabbits are less sensitive to infection than humans, they may represent an inexpensive alternative to primates for many studies.

Gene transfer in farm animals was initially aimed towards improving production efficiency, carcass quality (23), and disease resistance of livestock. However, it has been suggested that the simple over-expression of hormones such as growth hormone may have unacceptable side effects. Recently some elegant studies of growth using transgenic rats have been performed and are likely to yield valuable information on the biochemistry and physiology of growth (24, 25). A more successful application of transgenesis in farm animals has been the production of biomedically important proteins. The two most popular methods have been to direct expression to hematopoietic cells or to the lactating mammary gland. In the former case, transgenic swine expressing high levels of human hemoglobin were generated using the locus control region from the β-globin gene cluster to overcome positional effects and direct expression to the hematopoietic cells (26). However, due to its natural ability to synthesize and secrete large amounts of pro-

tein, the mammary gland has become the primary focus for the expression of heterologous proteins in large mammals. Transgene expression has been successfully directed to the mammary gland using promoter sequences from milk protein genes such as those encoding ovine β-lactoglobulin (BLG), goat β-casein, and murine whey acidic protein. The BLG promoter was used to direct expression of human α_1-antitrypsin in lines of transgenic mice and sheep (27). Interestingly, a wide variation in expression was observed between mouse lines, and from one lactation to another within a single line. In sheep however, similar high levels of heterologous protein were expressed in milk over consecutive lactations and over several generations in a given transgenic line, allowing the viable development of a flock of transgenic sheep. In separate studies high levels of expression of human tissue plasminogen activator were obtained in goat's milk under the control of the goat β-casein promoter (28). The development of suitable purification methods and the use of transgenically produced proteins in clinical trials are well advanced, and, if successful, will have important implications for the production of human proteins in transgenic livestock. Poor expression of the ovine promoter in the mouse may reflect species differences in recognizing heterologous versus homologous promoters and raises questions concerning the predictive value of mouse models. At best therefore the generation of transgenic mice may, in certain cases, only be a guide to the potential success of a transgene construct in another species.

Gene transfer could equally be used to enhance the quality and suitability of milk derived from domesticated animals as a food for human consumption. Human milk is devoid of β-lactoglobulin, which is responsible for most of the allergies to cows' milk, and has a relatively high content of lactoferrin, which is important in iron transport and combating bacterial infections. One could envisage in the future the reduction of saturated fat content in cows' milk and the knock-out of unwanted proteins or their replacement with other more useful components. Through the manipulation of milk constituents it should be possible to more closely emulate the desirable components of human milk. The alteration of milk composition would appear to be a practical possibility given that milk micelles are remarkably tolerant to changes in composition, as demonstrated by the knock-out of the mouse β-casein gene (29). Ethical concerns regarding the generation of transgenic animals, which have been engineered specifically for pharmaceutical, medical, or nutritional reasons, lie outside the scope of this overview, however it must be clearly ascertained that expression of a transgene does not compromise the animal.

Xenograft organs for transplantation surgery

The shortage of human organs for transplantation has raised interest in the possibility of xenotransplantation, i.e. the use of animal organs (30). However, the major barrier to successful xenogeneic organ transplantation is the phenomenon of complement-mediated hyperacute rejection (HAR), brought about by high levels of circulating natural antibodies that recognize carbohydrate determinants on the surface of xenogeneic cells. After transplantation of the donor organ, a massive inflammatory response ensues through activation of the classical complement cascade. This leads to activation and destruction of the vascular endothelial cells and, ultimately, the donor organ. The membrane-associated complement inhibitors, endogenous to the donor organ, are species restricted and thus confer only limited resistance. The complement cascade is regulated at specific points by proteins such as decay accelerating factor (DAF), membrane cofactor protein, and CD59. These regulators of complement activation are species specific. The initial strategy used to address HAR in porcine-to-primate xenotransplantation was to produce transgenic pigs expressing high levels of the human terminal complement inhibitor, hCD59. This was shown to protect the xenogeneic cells from human complement-mediated lysis in vitro (31). More recently, organ transplantation has been achieved using donor pigs which expressed human DAF on their endothelium (32), or both DAF and CD59 on erythrocytes, such that the proteins translocated to the cell membranes of endothelial cells (33). After transplantation, the pig hearts survived in recipient baboons for prolonged periods without rejection (33). Clearly, such genetic manipulations are bringing xenotransplantation ever closer to reality. If the isolation of suitable ES cells and application of homologous recombination becomes a reality in the pig, it may be possible to knockout the antigenic determinants to which antispecies antibodies bind, as a further strategy for eliminating HAR.

Summary

The use of nonmurine species for transgenesis will continue to reflect the suitability of a particular species for the specific questions being addressed, bearing in mind that a given construct may react very differently from one species to another. The application of transgenesis in the pig should produce major advances in the fields of transfusion and transplantation technology, while alterations in the composition of milk in a range of domesticated animals will have major effects on the production of pharmacologically important proteins and could eventually lead to the development of human milk substitutes. Despite the lack of germline transmission to date, major efforts continue to be directed towards the generation and use of ES cells from nonmurine species, using both traditional and new technologies, and the availability of such cells is likely to accelerate both the use of such species and the precision with which genetic changes can be introduced.

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Journal of Neuropathology and Experimental Neurology

Vol. 60, No. 2
February, 2001
pp. 195–207

Alzheimer-Associated Neuronal Thread Protein-Induced Apoptosis and Impaired Mitochondrial Function in Human Central Nervous System-Derived Neuronal Cells

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Abstract. In Alzheimer Disease (AD), dementia is due to cell loss and impaired synaptic function. The cell loss is mediated by increased apoptosis, predisposition to apoptosis, and impaired mitochondrial function. Previous studies demonstrated that the *AD7c-NTP* neuronal thread protein gene is over-expressed in AD beginning early in the course of disease, and that in AD, AD7c-NTP protein accumulation in neurons co-localizes with phospho-tau-immunoreactivity. To determine the potential contribution of AD7c-NTP over-expression to cell loss in AD, we utilized an inducible mammalian expression system to regulate *AD7c-NTP* gene expression in human CNS-derived neuronal cells by stimulation with isopropyl-1-β-D-thiogalacto-pyranoside (IPTG). IPTG induction of *AD7c-NTP* gene expression resulted in increased cell death mediated by apoptosis, impaired mitochondrial function, and increased cellular levels of the p53 and CD95 pro-apoptosis gene products as occur in AD. In addition, over-expression of AD7c-NTP was associated with increased levels of phospho-tau, but not amyloid-β immunoreactivity. These results suggest that AD7c-NTP over-expression may have a direct role in mediating some of the important cell death cascades associated with AD neurodegeneration, and further establish a link between AD7c-NTP over-expression and the accumulation of phospho-tau in preapoptotic CNS neuronal cells.

Key Words: Alzheimer disease; Apoptosis; Inducible gene expression; Mitochondria; Neuronal thread protein.

INTRODUCTION

Cerebral atrophy in Alzheimer disease (AD) has been linked to cell death and loss of synaptic terminals. Neuronal death in AD is mediated by apoptosis or enhanced predisposition to apoptosis (1–7) associated with increased expression of the p53, CD95 (Fas Receptor), Bax, and Bcl-x pro-apoptosis gene products (3, 8–10). Amyloid β peptide is neurotoxic (11–16) and can promote apoptosis by 1) activating pro-apoptosis genes or inhibiting anti-apoptosis genes (12, 17); 2) signaling through G-proteins (18); 3) promoting superoxide and oxidative free radical production (19–21); 4) synergistically acting with other neurotoxic or excitotoxic agents (22); 5) disrupting intracellular ion homeostasis; or 6) interacting with mutated presenilin to perturb cellular calcium regulation and promote oxidative stress (23, 24). However, apoptosis in sporadic AD may also be related to other underlying abnormalities that render cells more susceptible to oxidative stress and apoptosis since cell death in AD brains frequently occurs at a distance from the Aβ deposits (3, 6, 25). The roles of oxidative stress and free radical damage as major contributors to cell loss in AD as well as other neurodegenerative diseases (26–34) has become a major focus of investigation. Recent studies demonstrated that, in addition to apoptosis, mitochondrial DNA damage represents a second major factor contributing to cell loss and increased susceptibility to apoptosis in AD (35). The increased mitochondrial DNA damage in AD is associated with reduced mitochondrial mass and reduced mitochondrial enzyme gene expression (35). Impaired mitochondrial function could enhance neuronal sensitivity to apoptosis and result in the activation of pro-apoptosis signaling as occurs in AD (36, 37).

Previous investigations of gene expression abnormalities associated with early sporadic AD led to the identification of AD7c-NTP (NTP = neuronal thread protein) (38). *AD7c-NTP* is a novel cDNA that was isolated from a library prepared with mRNA extracted from AD temporal lobe tissue. The *AD7c-NTP* cDNA encodes a ~41 kD membrane-spanning protein in which subsequence analysis predicts the presence of a hydrophobic leader sequence, a myristoylation site, and17 cAMP, calmodulin-dependent protein kinase II, protein kinase C, or glycogen synthase kinase 3 phosphorylation sites (38). AD7c-NTP mRNA and protein levels are increased in brains with AD (38, 39). With regard to the typical neurodegenerative lesions, AD7c-NTP immunoreactivity was found co-localized with phospho-tau in neurons but unassociated with amyloid-β deposits. In addition, there appeared to be a reciprocal relationship between levels of AD7c-NTP and the levels of phospho-tau such that neurons with normal or only slightly altered cytomorphology exhibited intense degrees of AD7c-NTP immunoreactivity and low but detectably increased levels of phospho-tau, whereas the presence of mature, well-delineated phospho-tau-immunoreactive neurofibrillary tangles was associated with relatively low levels of AD7c-NTP (38). These results raised further questions regarding the potential sequence of events that leads to neuronal degeneration and the apparent link between

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Supported by AA-02666 and AA-10102 from the National Institutes of Health.

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AD7c-NTP accumulation in neurons and the eventual formation of phospho-tau immunoreactive neurofibrillary tangles. The present study demonstrates that over-expression of AD7c-NTP causes neuronal cell death mediated by apoptosis and impaired mitochondrial function with increased levels of p53, CD95, and phospho-tau as occur in AD.

MATERIALS AND METHODS

Cell Culture Conditions

PNET2 human CNS-derived neuronal cells (40) were used to study the effects of AD7c-NTP expression. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 4 mM glutamine, 10 mM nonessential amino acid mixture (Gibco-BRL, Grand Island, NY), and 9 g/l glucose (complete neuronal medium). Studies were done using cells seeded into 10^2 cm Petri dishes (2×10^5 cells/dish) or 96-well plates (2×10^4 cells/well).

Establishment of an Inducible Expression System to Study AD7c-NTP Over-Expression in Human CNS Neuronal Cells

The LacSwitch II inducible mammalian expression system (Stratagene, La Jolla, CA) was used to generate stably transfected PNET2 neuronal cell line clones in which *AD7c-NTP* gene expression was induced by stimulation with isopropyl-1-β-D-thiogalactopyranoside (IPTG) (1–5 mM). PNET2 cells were first stably transfected with the *pCMVLacII* vector in which expression of the Lac repressor protein was driven by a CMV promoter and targeted to the nucleus by the nuclear localization sequence. Stable Lac clones were maintained with hygromycin B (50 μg/ml). The clones were then transfected with a second vector (*pOPRSV1*) carrying the *AD7c-NTP* or chloramphenicol acetyltransferase (*CAT*) cDNA. The second vector contained an RSV promoter for driving gene expression and was equipped with ideal operator sequences for Lac repressor binding. Stable double transfectants were selected with hygromycin B (50 μg/ml) plus neomycin (G418; 400 μg/ml) and clones were isolated by the limiting dilution method (41). Isopropyl-1-β-D-thiogalactopyranoside (IPTG) stimulation (1–5 mM) turned off the Lac repressor protein and induced expression of CAT or AD7c-NTP within 8 hours (h). After induction, gene expression persisted for 48–96 h, and was rapidly inhibited by withdrawal of IPTG. In all experiments, IPTG was added once to the cultures, and the cells were analyzed 24–48 h later. CAT activity was measured as described previously (42). In a limited number of studies, stably transfected PNET2 cells induced to over-express the membrane-spanning human aspartyl asparaginyl β-hydroxylase (AAH) (43–45) were studied to determine if the effects of AD7c-NTP over-expression on neuronal viability could be due to overwhelming accumulation of the protein in the endoplasmic reticulum and attendant nonspecific activation of cell death cascades.

Measurement of Viability

Viability was measured by the crystal violet (46) assay using cells seeded into 96-well plates. After aspirating the medium, the cells were stained for 5 min with crystal violet solution (0.75% Crystal violet, 50% ethanol, 0.25% NaCl, 1.75% formaldehyde solution), then rinsed in distilled water. After air drying, the cells were lysed with 200 μl/well PBS containing 1% sodium dodecyl sulfate (SDS). The absorbances were measured in a Spectracount microplate reader (Packard Instrument Company, Meriden, CT). The crystal violet absorbances increased linearly with cell density between 10^4 and 5×10^5 cells/well.

Analysis of Apoptosis

To detect apoptosis, the cells were evaluated for genomic (Gn) DNA fragmentation laddering, Gn DNA nicking and fragmentation using a quantitative solution-based assay, and nuclear pyknosis and karyorhexis. To isolate Gn DNA, cells grown in 10 cm^2 dishes were dounce homogenized in buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, and 0.25 M sucrose. Nuclei were pelleted by centrifuging the samples at 5000× g for 15 min at 4°C. GnDNA was isolated from the nuclear pellets using 7 M guanidine HCl and Wizard Maxi-prep resin (Promega, Madison, WI) according to the manufacturer's protocol. DNA concentration and purity were assessed from the 260 nm and 280 nm absorbance values. To detect DNA fragmentation ladders, 10-μg samples of GnDNA were electrophoresed in 3% agarose gels. The DNA stained with ethidium bromide and visualized with ultraviolet illumination. The degree of DNA fragmentation or nicking was assessed by measuring [α-^{32}P]dCTP incorporation in reactions devoid of oligonucleotide primers (47, 48). Paired, duplicate 100 ng samples of genomic DNA were incubated for 15 min at 22°C in 20 μl reactions containing 1× Klenow buffer, 1 U Klenow, and 0.2 μCi of [α-^{32}P]dCTP. Labeled DNA was isolated using Wizard DNA Clean-up resin (Promega), and [α-^{32}P]dCTP incorporation was measured in a scintillation counter.

Hoechst's H33258 staining was used for in situ detection of nuclear karyorrhexis and pyknosis associated with apoptosis. The cells were fixed with Histochoice solution (Amresco, Solon, Ohio), permeabilized with 0.05% saponin in PBS. Replicate cultures were stained for 5 min at room temperature with 1 μg/ml Hoechst H33258 in PBS. After rinsing in PBS, the labeled cells were preserved under coverglass with Vectashield (Vector Laboratories, Burlingame, CA) and examined by fluorescence microscopy.

Analysis of Mitochondrial Function

Mitochondrial function was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (49) assay. The MTT assay is based upon the conversion of MTT to formazan by mitochondrial dehydrogenases. The MTT assay was performed with cells seeded into 96-well plates at a density of 2×10^4 cells/well. Three hours prior to harvesting, the cells were labeled with MTT (500 μg/ml). The cells were then rinsed briefly in PBS and lysed in acidic isopropanol (0.04 N HCl in isopropanol; 100 μl/well). The absorbances were measured in a Spectracount microplate reader (Packard Instrument Company). The MTT absorbances increased linearly with cell density between 10^4 and 5×10^5 cells/well.

Mitochondrial mass and mitochondrial functions were evaluated using MitoTracker mitochondria-specific cell permeable dyes (Molecular Probes, Eugene, OR). MitoTracker Green FM

labels mitochondria irrespective of oxidative activity and therefore is used to assess mitochondrial mass. MitoTracker Red (CM-H_2Xros) accumulates only in metabolically active mitochondria and the reduced dihydrotetramethyl rosamine is rendered fluorescent via oxidation within the mitochondria. Cells grown in 96-well plates were labeled with incubated fore 15 min with MitoTracker Red or MitoTracker Green FM according to the manufacturer's instructions. The cells were rinsed in TBS and fluorescence light units were measured with a Fluorocount plate reader (Packard Instrument Company). Subsequently, the cells were stained with H33258 to determine cell density using the Fluorocount plate reader since H33258 fluorescence intensity increases linearly with cell number between 1×10^4 and 5×10^5 cells per well (data not shown). MitoTracker Red/H33258 and MitoTracker Green/H33258 ratios were used as indices of mitochondrial function and mitochondrial mass, respectively.

Protein Expression

Protein expression was assessed by Western blot analysis (41) and the Microtiter Immunocytochemical ELISA (MICE) Assay (50). Western blot analysis was used to measure protein expression in cell lysates prepared in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (41). Protein concentrations were measured using the BCA assay (Pierce Chemical Company, Rockford, IL). Samples containing 60 μg of protein were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to PVDF membranes, and analyzed by Western immunoblotting (51–53). Immunoreactivity was detected with horseradish peroxidase conjugated secondary IgG and PicoWest enhanced chemiluminescence reagents (Pierce Chemical Company), and quantified using the Kodak Digital Imager System.

The MICE assay is a rapid and sensitive method of quantifying immunoreactivity in 96-well micro-cultures and combines the advantages of the enzyme-linked immunosorbent assay with immunocytochemical staining to permit in situ quantification of protein expression with values normalized to cell density (50). Briefly, the cells were fixed overnight in Histochoice (Amresco), permeabilized with 0.05% saponin in Tris-buffered saline (50 mM Tris, pH 7.5, 0.9% NaCl; TBS), and blocked with Superblock-TBS (Pierce Chemical Company). The cells were then incubated overnight (10–12 h) at 4°C with primary antibody (0.5–1 μg/ml) diluted in TBS containing 0.05% Tween-20 and 0.5% bovine serum albumin (TBST-BSA). Antibody binding was detected with horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG (Pierce Chemical Company) diluted 1:10,000 in TBST-BSA (30-min incubation at room temperature). Immunoreactivity was revealed with TMB (100 μl/well) soluble peroxidase substrate (Pierce Chemical Company). Reactions were stopped by the addition of 100 μl/well of 1 M H_2SO_4, and the absorbances were measured at 450 nm using an automated ELISA reader. Between incubations, the cells were washed 3 times (2 min per wash) in TBS with gentle platform agitation.

To compare the levels of protein expression it was necessary to correct for differences in cell density. After measuring immunoreactivity, the plates were washed in TBS and the cells were stained 0.1% Coomassie blue dye in 40% methanol/10% acetic acid. After extensively washing the plates in water, the dye was eluted with 1% SDS in PBS (200 μl/well). The absorbances (560 nm) were measured using a Spectracount plate reader (Packard Instrument Company). The MICE index was calculated from the ratio of the absorbances measured for immunoreactivity and cell density. Coomassie blue absorbance increases linearly with cell density between 1×10^4 and 5×10^5 cells per well. Eight or16 replicate culture wells were analyzed in each experiment. All experiments were repeated at least 3 times.

Statistical Analysis

Data depicted in the graphs represent the mean ± SD generated with results obtained from 3 to 6 experiments. Intergroup comparisons were made using Student *t*-tests or analysis of variance (ANOVA) with Fisher least significant difference (LSD) post-hoc tests. Statistical analysis was performed using the Number Cruncher Statistical System (Dr. Jerry L. Hintze, Kaysville, UT).

RESULTS

Over-Expression of AD7c-NTP Using the LacSwitch Inducible System

PNET2 human CNS-derived neuronal cells were used to study the effects of AD7c-NTP expression. In preliminary studies, we observed that constitutive over-expression of AD7c-NTP caused progressive neuronal cell death, which prohibited the establishment of stably transfected cell lines. To circumvent this problem, we used the LacSwitch II inducible mammalian expression system (Stratagene) in which *AD7c-NTP* gene expression was regulated by IPTG stimulation (1–5 mM). With this 2-vector system, expression of the Lac repressor protein was driven by a CMV promoter, while expression of the AD7c-NTP or chloramphenicol acetyltransferase (CAT) cDNA was driven by an RSV promoter that contained operator sequences for Lac repressor protein binding. In the absence of IPTG, high level Lac repressor protein expression blocked transcription of *AD7c-NTP* or *CAT*, while stimulation with 1–5 mM inhibited Lac repressor protein expression and permitted the expression of CAT or AD7c-NTP. Gene expression was induced within 8 h of IPTG exposure, and persisted for 48–96 h. Withdrawal of IPTG resulted in inhibition of gene expression within 8–12 h.

Among the 556 doubly transfected clones that were isolated, we identified 8 that were suitable for study in that the basal (unstimulated) levels of *AD7c-NTP* or *CAT* gene expression were virtually undetectable, and IPTG-induced expression was increased by at least 2-fold above background. Representative results obtained from 2 CAT control clones (Lac A-CAT; Lac B-CAT) and 2 AD7c-NTP-expressing clones (Lac B-AD7c; Lac F-AD7c) are presented. Western blot analysis using the N3I4 monoclonal antibody generated to recombinant AD7c-NTP (38, 39) detected a single ~41 kD protein in cells that

over-expressed the *AD7c-NTP* gene. As illustrated in Figure 1A and 1B, stimulation of LacF-AD7c cells with 3 or 4 mM IPTG resulted in sharply increased levels of AD7c-NTP protein expression by Western blot analysis and densitometry. Similar results were obtained using Lac B-AD7c cells, although the peak levels of AD7c-NTP expression were 50% lower than in LacF-AD7c cells (data not shown).

The MICE assay was also used to measure AD7c-NTP because the assay is more sensitive and permits analysis of multiple replicate cultures in each experiment. The MICE assay studies demonstrated increased levels of N3I4-immunoreactive AD7c-NTP in Lac B-AD7c and Lac F-AD7c cells, but not in either Lac A-CAT or Lac B-CAT cells (Fig. 2A). At the lower concentrations of IPTG (1–2 mM), there was a slightly graded response with progressive increases in AD7c-NTP expression. The graded effect of IPTG was likely due to the greater sensitive of the MICE assay compared with Western blot analysis since very low levels of immunoreactivity can be detected in as few as 10^4 cells (50). However, peak levels of AD7c-NTP expression were consistently achieved with 3 or 4 mM IPTG stimulation as detected by both the MICE assay and Western immunoblotting.

The CAT assay was used to demonstrate IPTG-induced gene expression in Lac A-CAT and Lac B-CAT cells. As illustrated in Figure 2B, IPTG stimulation of Lac A-CAT and Lac B-CAT cells resulted in strikingly increased levels of CAT activity. Peak-level induction of CAT activity occurred with 3 mM IPTG stimulation. CAT activity was not detected in IPTG-stimulated Lac B-AD7c Lac F-AD7c cells. AAH expression was low-level in uninduced cells and abundant after 3 mM IPTG stimulation as demonstrated by Western blot analysis (data not shown).

Reduced Viability with AD7c-NTP Over-Expression

IPTG-induced expression of AD7c-NTP resulted in increased cell death associated with the gradual accumulation of round, refractile floating cells that failed to exclude Trypan blue dye. In contrast, cells that over-expressed either the CAT or AAH gene remained flat, polygonal, and had delicate cell processes. To quantify the effects of AD7c-NTP over-expression on neuronal cell viability, Lac B-AD7c, Lac F-AD7c, Lac A-CAT, Lac B-CAT, and Lac A-AAH cells were seeded into 96-well plates at the he same viable cell density (2×10^4/well). Beginning 8 h after seeding when the cells were firmly adhered to the surfaces, the cultures were treated with 0 or 3 mM IPTG. After 24 h of IPTG stimulation, viability was measured using the Crystal violet assay. As shown in Figure 3, the Lac A-CAT, Lac B-CAT, and Lac A-AAH cultures had similar viable cell densities independent of IPTG exposure, whereas the Lac B-AD7c and Lac F-AD7c cells manifested significant reductions in viability with IPTG stimulation ($p < 0.005$).

AD7c-NTP-Induced Apoptosis

To determine if the increased cell death associated with AD7c-NTP over-expression was mediated by apoptosis, the cells were stained with Hoechst H33258 dye, and genomic DNA was analyzed for fragmentation and nicking after 48 h of IPTG induction of gene expression. H33258 is a DNA binding dye that detects nuclear morphology including karyorrhexis and pyknosis associated with apoptosis. H33258 staining demonstrated uniform nuclear labeling in IPTG stimulated (3 mM) Lac A-CAT and Lac B-CAT cells (Fig. 4A) and unstimulated Lac B-AD7c and Lac F-AD7c cultures (data not shown). In contrasts, the IPTG stimulated Lac B-AD7c and Lac F-AD7c cultures exhibited increased nuclear karyorrhexis and pyknosis (Fig. 4B), consistent with apoptosis. Agarose gel electrophoresis and ethidium bromide staining of GnDNA isolated from the cells demonstrated only high molecular weight DNA in Lac A-CAT and Lac B-CAT cells, independent of IPTG stimulation, and in unstimulated Lac B-AD7c and Lac F-AD7c cultures (Fig. 4C). In contrast, the IPTG-stimulated Lac B-AD7c and Lac F-AD7c cultures exhibited increased Gn DNA fragmentation laddering characteristic of apoptosis (Fig. 4C). The third assay used to demonstrate increased Gn DNA fragmentation in cells that over-expressed AD7c-NTP involved quantification of [α^{32}P]dCTP incorporated into the 3′ ends of fragmented or nicked DNA. Corresponding with the predominantly high molecular weight GnDNA detected by agarose gel electrophoresis, the Lac A-CAT and Lac B-CAT cells, with or without IPTG stimulation, exhibited low DNA labeling indices, while IPTG induction of AD7c-NTP resulted in significantly increased [α^{32}P]dCTP incorporation into Gn DNA ($p < 0.001$) (Fig. 4D).

Impaired Mitochondrial Function Associated with AD7c-NTP Over-Expression

In AD, neuronal cell death is mediated by both apoptosis and impaired mitochondrial function (27, 35). To determine if AD7c-NTP over-expression could lead to impaired mitochondrial function, we examined mitochondrial function and mitochondrial mass in IPTG-stimulated (3 mM) Lac A-CAT, Lac B-CAT, Lac B-AD7c, and Lac F-AD7c cells using the MTT assay and MitoTracker fluorescent dye labeling. The MTT labeling studies demonstrated similarly high levels of MTT activity in Lac-CAT control cells, independent of IPTG stimulation (Fig. 5). In contrast, the Lac B-AD7c and Lac F-AD7c cultures manifested significantly reduced levels of MTT activity with IPTG stimulation (Student *t*-test analysis; $p < 0.005$). Moreover, Lac F-AD7c cells had reduced levels of MTT activity in the absence of IPTG exposure, despite the normal levels of viability and undetectable AD7c-NTP expression (Fig. 5), suggesting some degree of leakiness in the regulation of gene expression in those cells.

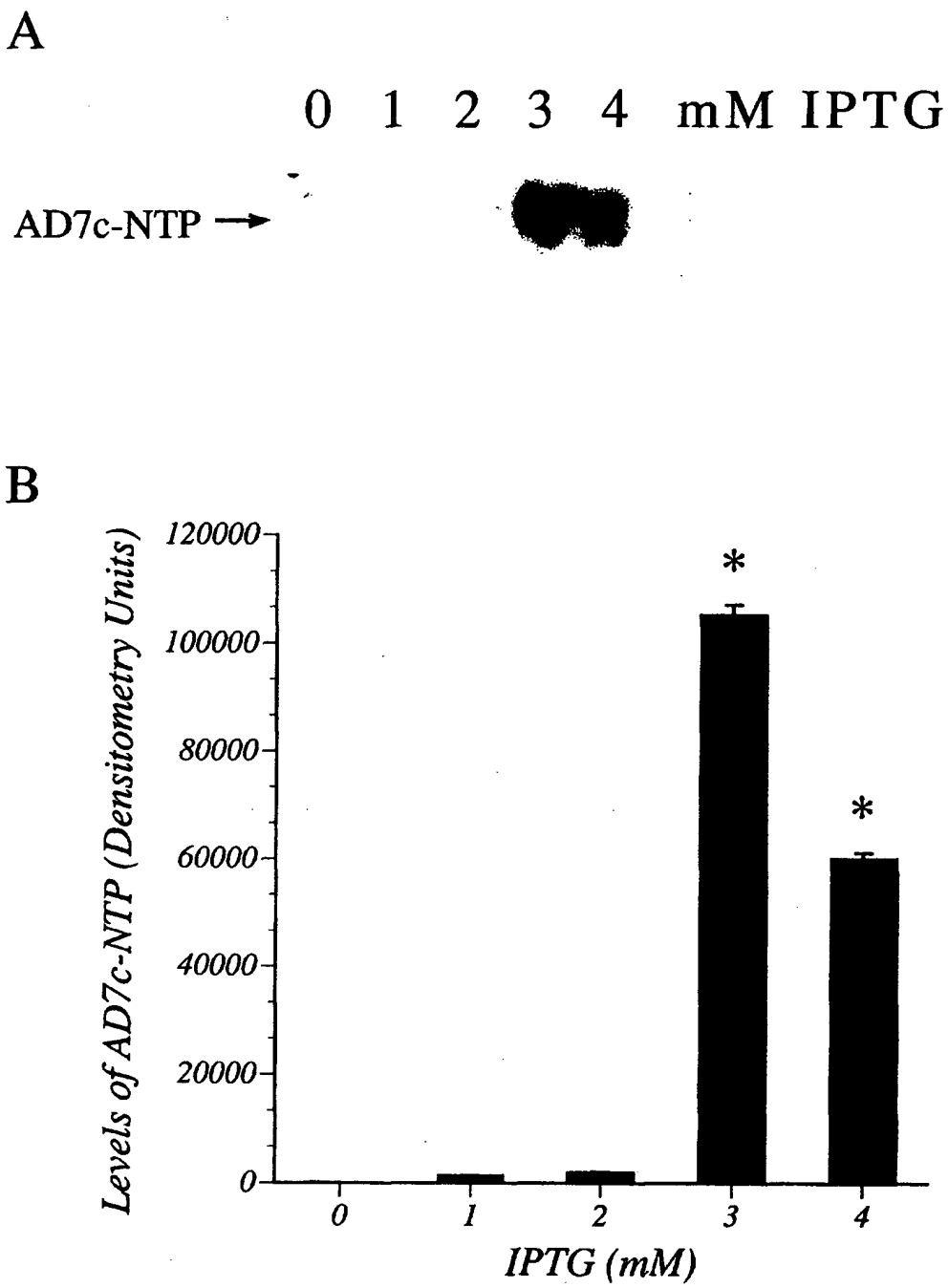


Fig. 1. Regulation of AD7c-NTP expression by IPTG stimulation of PNET2 cells that were stably transfected with the LacSwitch II vector system. PNET2 cells were stimulated for 24 h with 0–4 mM IPTG. Cell lysates, each containing 60 μg of protein, were subjected to Western blot analysis, and AD7c-NTP protein expression was quantified by densitometry. Panel (A) depicts a representative Western blot autoradiograph in which AD7c-NTP immunoreactivity was detected using the N3I4 monoclonal antibody. The approximate molecular mass of the protein indicated by the arrow is ~41 kD. Panel (B) depicts the results (mean ± SD) of densitometric analysis of 3 Western blot autoradiographs used to measure the levels of AD7c-NTP protein expression in cells exposed to 0–4 mM IPTG for 24 h. Asterisks indicate results that are significantly different from control (0 IPTG) ($p < 0.001$).

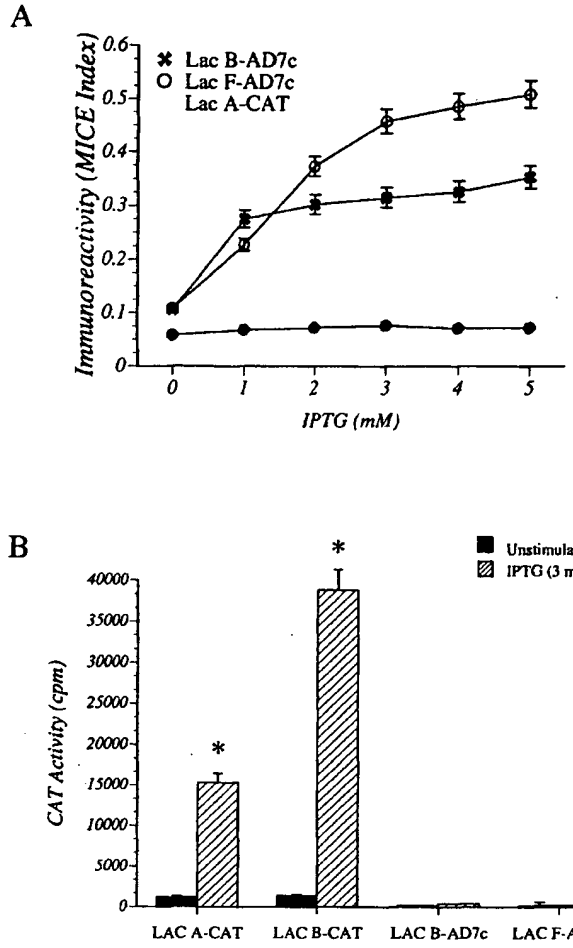


Fig. 2. Regulation of AD7c-NTP expression (A) or chloramphenicol acetyltransferase (CAT) activity (B) by IPTG stimulation of PNET2 cells that were stably transfected with the LacSwitch II vector system. A: Lac B-AD7c, Lac F-AD7c, and Lac A-CAT PNET2 clones were stimulated for 24 h with 0–5 mM IPTG. AD7c-NTP immunoreactivity was measured using the MICE assay and the N3I4 monoclonal antibody. The MICE index reflects levels of immunoreactivity corrected for cell density. The values plotted represent mean ± SD of AD7c-NTP expression measured in 16 replicate culture wells. B: Lac B-AD7c, Lac F-AD7c, and Lac A-CAT PNET2 clones were stimulated for 24 h with 0 or 3 mM IPTG, and the cell lysates were analyzed for CAT activity using [^{14}C]Acetyl Coenzyme A as the substrate. The graph depicts the mean ± SD of CAT activity measured in 6 replicate cultures. Similar results were obtained in 3 repeated experiments. Asterisks indicate results that are significantly different from control (0 IPTG) ($p < 0.001$). In addition, the MICE assay results shown for the Lac B-AD7c and Lac F-AD7c transfected cells were significantly different from the Lac A-CAT control cells stimulated with 1–5 mM IPTG.

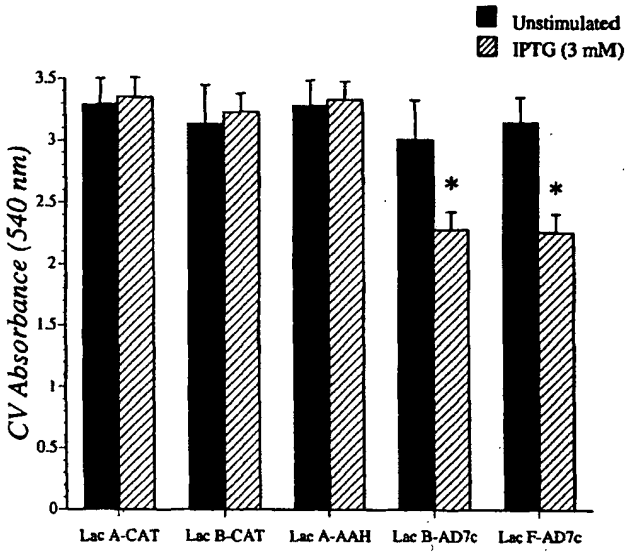
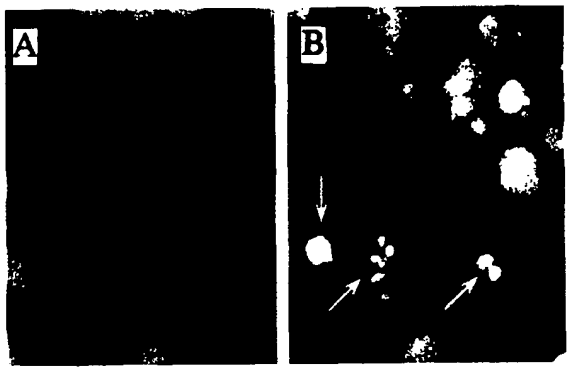


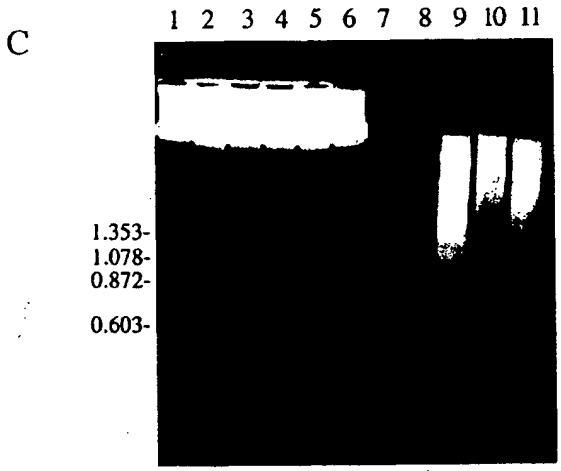
Fig. 3. Reduced viability of PNET2 neuronal cells induced to express AD7c-NTP. Lac B-AD7c, Lac F-AD7c, Lac A-CAT, and Lac A-AAH PNET2 cells were seeded into 96-well plates (2×10^4 viable cells per well). The cells were treated with 0 or 3 mM IPTG for 24 h and then analyzed for viable cell density using the Crystal violet assay. Crystal violet absorbance was linearly correlated with cell densities between 10^4 and 5×10^5 cells per well (data not shown). Asterisks indicate significant differences from corresponding control, unstimulated cultures by ANOVA ($p < 0.005$).

MitoTracker Red and MitoTracker Green fluorescence labeling studies were used to assess mitochondrial function and mitochondrial mass, respectively. Parallel cultures were labeled with MitoTracker Red, MitoTracker Green, or both dyes and fluorescence intensity was measured in a Fluorocount microplate reader. The single-labeled studies were performed to confirm results obtained by double-labeling, in the event that intense degrees of labeling resulted in incorrect detection of fluorescence emission. The cells were subsequently labeled with H33258 to assess cell density using the Fluorocount microplate reader. The calculated ratios of MitoTracker Red/H33258, MitoTracker Green/H33258, and MitoTracker Red/MitoTracker Green were used to compare labeling indices among the cultures. These studies demonstrated significantly reduced levels of MitoTracker Red/H33258 ($p < 0.001$), MitoTracker Green/H33258 ($p < 0.001$), and MitoTracker Red/MitoTracker Green ($p < 0.01$) in IPTG-stimulated (3 mM) Lac B-AD7c and Lac F-AD7c cultures relative to the Lac A-CAT and Lac B-CAT cells (Fig. 6A, B). Fluorescence microscopy demonstrated bright labeling throughout the perikarya and cell processes of IPTG stimulated Lac-CAT control cells and diffusely reduced labeling of cells that over-expressed AD7c-NTP (Fig. 6C–F).

AD7c-NTP-Induced Pro-Apoptosis Gene Expression

In AD, apoptosis is associated with increased levels of p53 and CD95/Fas-Apo-1 receptor (3, 4, 10). To determine





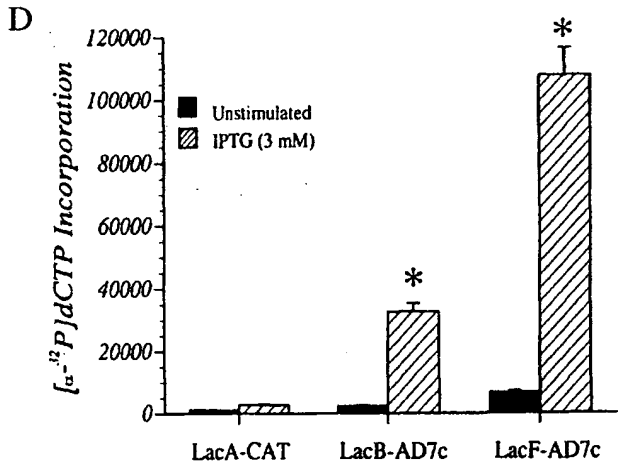


Fig. 4. AD7c-NTP-induced apoptosis. Lac B-AD7c, Lac F-AD7c, and Lac A-CAT PNET2 cells were stimulated with IPTG for 48 h. Apoptosis was assessed by Hoechst H33258 staining (A, B), agarose gel electrophoresis and ethidium bromide staining of isolated genomic DNA (C), and measurement of [α-^{32}P]dCTP incorporation into the 3′ ends of nicked or fragmentedgenomic DNA (D). IPTG-stimulated Lac A-CAT-expressing PNET2 clones (A) exhibited uniform nuclear labeling, while

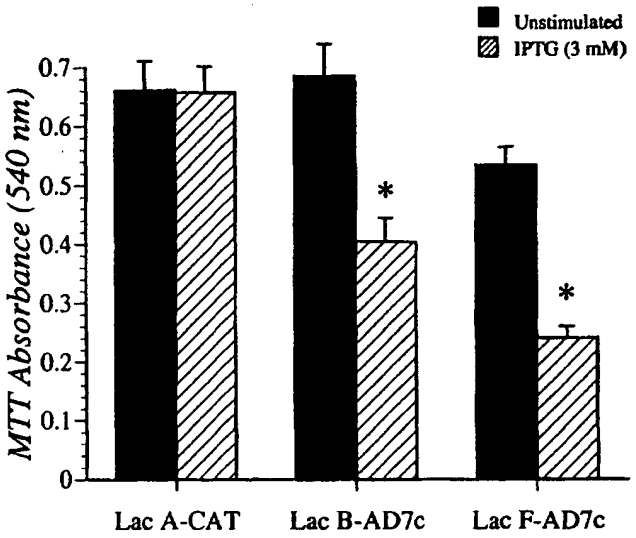


Fig. 5. Reduced MTT activity after induction of AD7c-NTP expression. The MTT assay provides a measure of mitochondrial function since conversion of MTT to its purple formazan precipitate is mediated by mitochondrial dehydrogenases. Lac B-AD7c, Lac F-AD7c, and Lac A-CAT PNET2 cells were seeded into 96-well plates (2×10^4 viable cells per well). The cells were treated with 0 or 3 mM IPTG for 24 h and then analyzed for MTT activity. MTT absorbance was linearly correlated with cell densities between 10^4 and 5×10^5 cells per well (data not shown). Asterisks indicate significant differences from corresponding control, unstimulated cultures by Student *t*-test analysis ($p < 0.005$). Note the reduced levels of MTT activity in the unstimulated Lac F-AD7c cells, despite control levels of viability as illustrated in Figure 3.

if apoptosis associated with AD7c-NTP occurs by a similar mechanism, cells stimulated with 0–5 mM IPTG were analyzed for p53 and CD95 expression using the MICE assay to quantify immunoreactivity directly in the cultured

←

the Lac F-AD7c (B) and Lac B-AD7c-expressing cells manifested increased nuclear condensation and fragmentation (arrows) by H33258 dye staining. C: Genomic DNA samples isolated from Lac F-AD7c- and Lac A-CAT-expressing cells were electrophoresed in a 3% agarose gel, stained with ethidium bromide, and photographed under UV light (Lane 1-unstimulated Lac F-AD7c; Lane 2-unstimulated Lac A-CAT; Lanes 3–6: 1, 2, 3, or 4 mM IPTG-stimulated Lac A-CAT; Lane 7: DNA molecular weight reference ladder. Bars on the left side indicate the sizes (kbp) of the visible bands. Lanes 8–11: 1, 2, 3, or 4 mM IPTG-stimulated Lac F-AD7c). D: Quantification of [α-^{32}P]dCTP incorporated into the 3′ ends of nicked or fragmented genomic DNA isolated from cells exposed to 0 or 3 mM IPTG for 48 h. Samples of 100 ng of purified DNA were used in a primer-independent end-labeling assay. [α-^{32}P]dCTP incorporation was measured in a scintillation counter. The graphed data depict results obtained from 6 replicate samples in each group. Asterisks indicate significant differences from unstimulated control cultures by ANOVA and Fisher LSD post hoc testing * $p < 0.001$. All experiments were repeated at least 3 times.

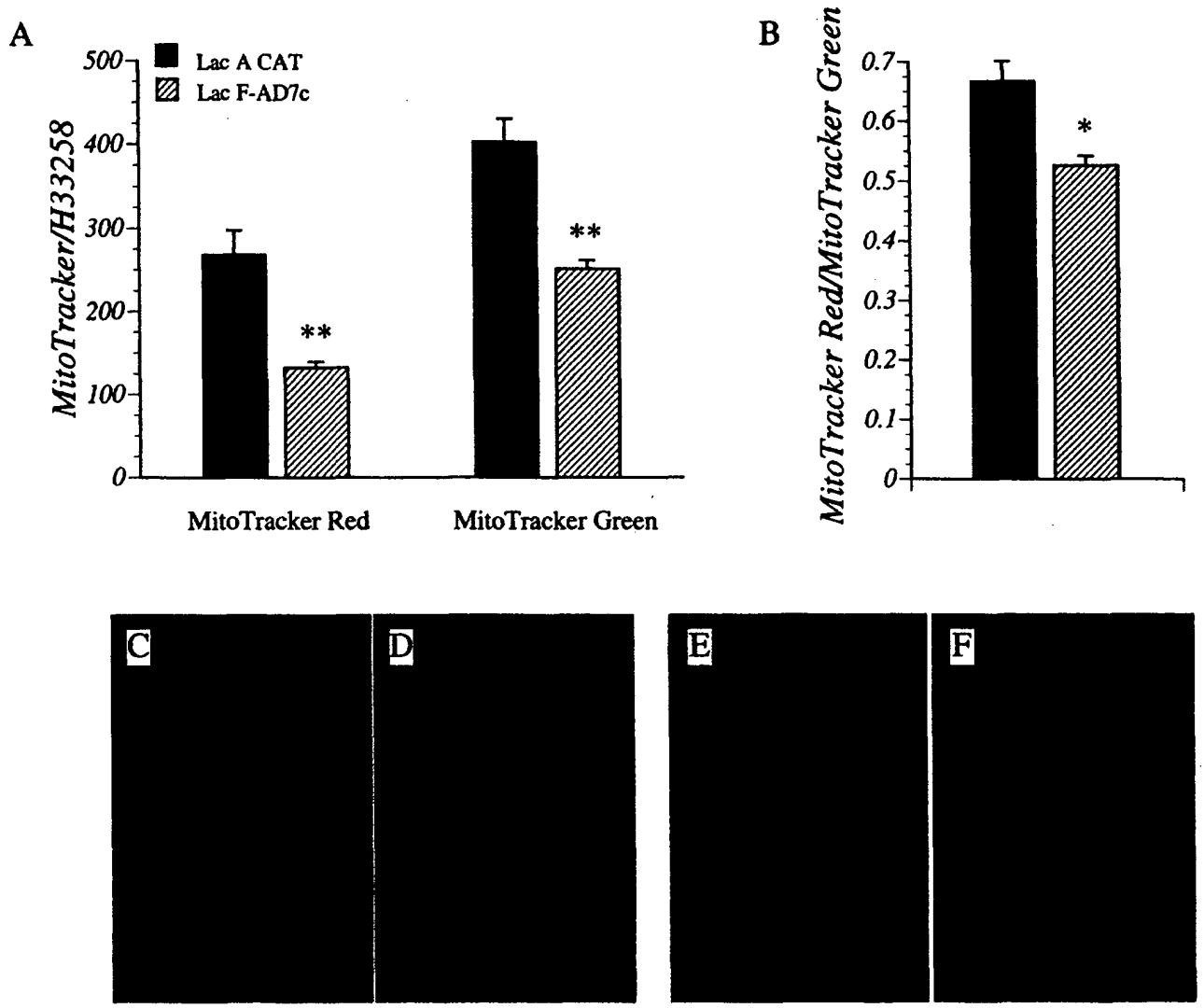


Fig. 6. Reduced mitochondrial function and mitochondrial mass with IPTG stimulation of AD7c-NTP expression demonstrated using MitoTracker labeling. The intensity of MitoTracker Red labeling corresponds with the levels of oxidative enzyme activity. In contrast, MitoTracker Green labels all mitochondria, irrespective of functional integrity, and therefore the labeling intensity corresponds to mitochondrial mass. Lac F-AD7c, and Lac A-CAT PNET2 cells were seeded into 96-well plates (2×10^4 viable cells per well). The cells were treated with 0 or 3 mM IPTG for 24 h and then labeled with MitoTracker Red or MitoTracker Green fluorescent dye. Fluorescence intensity was measured in a Fluorocount fluorescence microplate reader (MitoTracker Green: Ex 485 nm; Em 530 nm; MitoTracker Red: Ex 540 nm; Em 590 nm). Subsequently, the cells were labeled with H33258 dye and Fluorescence intensity (Ex 360 nm; Em 460 nm) was again measured to assess cell density. The MitoTracker/H33258 ratios were calculated to adjust the measured MitoTracker labeling intensity to cell density. Graphs in Panel (A) reflect the MitoTracker labeling indices for IPTG-stimulated Lac A-CAT and Lac F-AD7c cells. Panel (B) shows the mean ± SD of MitoTracker Red/MitoTracker Green ratios in double-labeled cells. This graph depicts the relative abundance of function (MitoTracker Red) to total (MitoTracker Green) mitochondria in the cultures. Asterisks indicate significant differences from control by Student *t*-test analysis (* $p < 0.01$; ** $p < 0.001$). C–F: Fluorescence microscopy of MitoTracker Red (C, D) or MitoTracker Green (E, F) labeled Lac A CAT (C, E) or Lac F-AD7c (D, F) cells stimulated with 3 mM IPTG for 24 h. The MitoTracker dyes label mitochondria distributed throughout the cytoplasm and cell processes.

cells. In addition, since phospho-tau immunoreactivity co-localizes with AD7c-NTP in AD brains (39), and amyloid-β peptide has been linked to apoptotic neuronal cell death and is likely to play a role in AD neurodegeneration (11, 13, 17, 54–56), parallel cultures were analyzed for phospho-tau (Alz-50; obtained from Dr. Peter Davies) amyloid-β (A4; Dako Corp) immunoreactivity to determine if cellular accumulation of these molecules occurred with

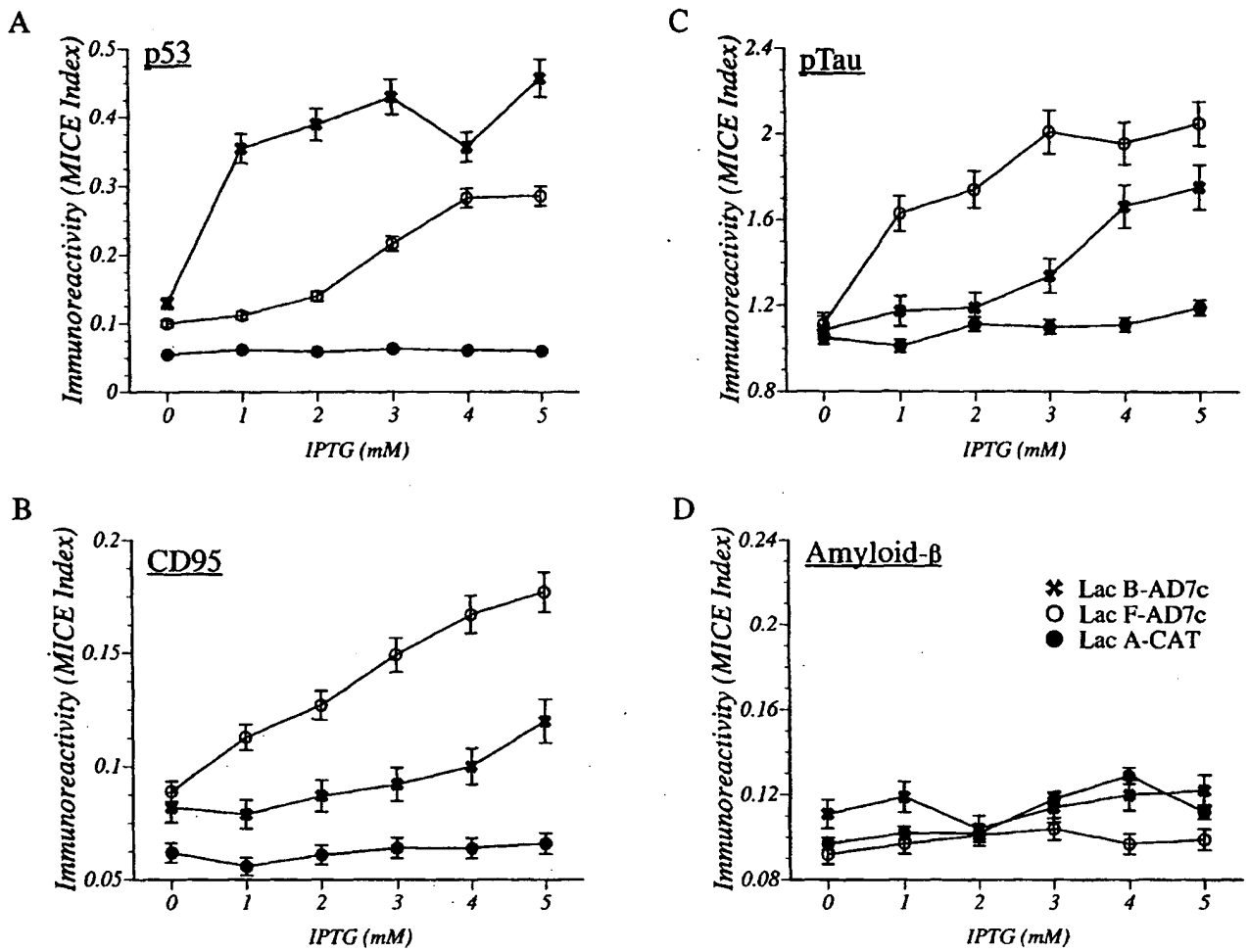


Fig. 7. Increased levels of p53 (A), CD95 (Fas Receptor) (B), and phospho-(p) Tau (C), but not amyloid-β (D) immunoreactivity with AD7c-NTP expression. Lac B-AD7c, Lac F-AD7c, and Lac A-CAT PNET2 cells were stimulated with 0–5 mM IPTG for 24 h. Immunoreactivity was quantified directly in cultured cells (96-well plates) using the microtiter immunocytochemical ELISA (MICE) assay. The MICE indices represent levels of immunoreactivity corrected for cell density (see Materials and Methods). Graphs depict mean ± SD of immunoreactivity levels detected in 24 replicate culture wells. All experiments were repeated at least 3 times with similar results. The differences between the control and experimental groups were statistically significant with respect to p53, CD95, and pTau in cultures stimulated with 3–5 mM IPTG ($p < 0.01$).

AD7c-NTP over-expression. In the absence of IPTG stimulation, expression of p53, CD95, phospho-tau, and amyloid-β were low-level in all cultures. With IPTG stimulation, the levels of p53 and CD95 increased in the Lac B-AD7c and Lac F-AD7c cultures, but not in the Lac-CAT control cells (Fig. 7). Although the levels of p53 and CD95 did not exactly parallel the levels of AD7c-NTP expression (Fig. 2A), the levels generally increased with IPTG concentration. In addition, parallel increases in the levels of phospho-tau immunoreactivity occurred in cells that over-expressed *AD7c-NTP* gene (Fig. 7C), whereas the levels of amyloid-β remained unchanged with IPTG-induction of either *AD7c-NTP* or *CAT* gene expression (Fig. 7D).

DISCUSSION

Increased levels of AD7c-NTP protein are detectable in the brain and cerebrospinal fluid at relatively early stages of AD. Abundant intra-neuronal AD7c-NTP protein expression is predominantly localized in either mildly degenerating or cytologically intact-appearing neurons that have preneurofibrillary tangle phospho-tau accumulation (39). Although initial studies suggested that AD7c-NTP over-expression might contribute to AD neurodegeneration by promoting cell death (38), we were unable to investigate this issue using standard stably transfected cells because of progressive depletion of the cells in culture, which probably died due to apoptosis induced by

AD7c-NTP expression. To circumvent this problem, we utilized the Lac Switch II inducible mammalian expression system in which AD7c-NTP expression was negatively regulated by a Lac repressor protein, and induced by IPTG stimulation, which inhibited Lac repressor gene expression. Thus, in the absence of IPTG, gene expression was kept off, but with IPTG stimulation, gene expression was rapidly induced and sustained for up to 96 h, after which the cells become refractory to IPTG (Stratagene). Using the Lac Switch II vector system we isolated, as expected, relatively few (8 of 556) doubly transfected clones in which gene expression was tightly regulated and associated with moderately high levels of AD7c-NTP protein expression or CAT activity. The studies reported herein were conducted using 2 representative clones that expressed AD7c-NTP and 2 clones that expressed the CAT gene.

IPTG stimulation resulted in increased expression of AD7c-NTP or CAT activity, which was readily detected within 24 h by Western blot analysis or measurement of CAT activity, respectively. Over-expression of the AD7c-NTP gene resulted in significantly reduced neuronal cell viability as demonstrated with the Crystal violet assay. Further studies demonstrated that AD7c-NTP-induced neuronal death was mediated by apoptosis, characterized by increased densities of cells with karyorrhectic or pyknotic nuclei, DNA fragmentation laddering, and incorporation of [α-^{32}P]dCTP into the 3′ ends of nicked or fragmented genomic DNA. In AD, apoptosis is also associated with nuclear fragmentation and pyknosis, genomic DNA laddering, and increased genomic DNA nicking and fragmentation as demonstrated with quantitative and in situ end-labeling assays (3, 35). This suggests that over-expression of AD7c-NTP may be sufficient to cause apoptosis in CNS neuronal cells. Although cell types other than neurons exhibit increased apoptosis and pro-apoptosis gene expression with AD and other forms of neurodegeneration (3, 4), the mechanisms of non-neuronal cell degeneration are unknown and beyond the scope of these investigations.

In AD brains, the levels of AD7c-NTP protein detected by Western blot analysis or the ELSIA are typically only 2- or 3-fold higher than control (38), whereas in the cells used in these experiments, the N3I4-immunoreactive ~41 kD AD7c-NTP species was abundantly expressed in IPTG-stimulated AD7c-NTP transfected cells, and virtually undetectable in unstimulated cells or in cells that were induced to express the CAT gene. Since the AD7c-NTP measurements were made in cell lysates or directly in cultured cells using the MICE assay, all of the AD7c-NTP immunoreactivity detected was intracellular in origin (as opposed to secreted). Moreover, it is noteworthy that at later stages of AD, the levels of AD7c-NTP protein measured in brain tissue extracts or in CSF do not necessarily correlate with severity of dementia, yet in all cases of advanced AD, high levels of AD7c-NTP protein accumulation is detectable in cortical neurons by immunohistochemical staining (38, 39). These observations suggest that the neuronal cells at greatest risk for undergoing AD7c-NTP-induced apoptosis are those that express high levels of AD7c-NTP protein with intracellular accumulations of the protein.

This study demonstrated that AD7c-NTP-induced neuronal cell death was also associated with impaired mitochondrial function. The MTT assay provides a measure of mitochondrial function because the conversion of MTT to formazan is mediated by mitochondrial dehydrogenases. In cells that over-expressed AD7c-NTP, MTT activity was significantly reduced relative to control cells that were induced to express the *CAT* gene. In addition, using MitoTracker fluorescent dyes, we detected reduced levels of MitoTracker Red and MitoTracker Green fluorescence associated with AD7c-NTP over-expression. The MitoTracker Red dye provides a measure of mitochondrial function because the dye is rendered fluorescent via oxidation within metabolically active mitochondria. The reduced levels of MitoTracker Red fluorescence corroborate the results of the MTT assay. In addition, the MitoTracker Green fluorescence labeling studies revealed reduced mitochondrial mass, after correcting for cell density. The MitoTracker Green labeling index provides a reliable measurement of mitochondrial mass because the dye labels mitochondria irrespective of oxidative activity. The reductions in MTT activity and MitoTracker Red fluorescence were not due to cell death because in parallel cultures, the cells were demonstrated to be viable using the Crystal violet assay. Moreover, only adherent cells were analyzed by the MTT and Mitotracker fluorescence assays since nonviable PNET2 cells are nonadherent and easily removed during the post-labeling rinses. Therefore, these studies demonstrate that AD7c-NTP over-expression results in impaired mitochondrial function as well as reduced mitochondrial mass.

In AD, neuronal loss can be mediated by apoptosis, increased predisposition to apoptosis, or impaired mitochondrial function (27, 33–35, 57). In AD, impaired mitochondrial function characterized by reduced levels of cytochrome oxidase expression is accompanied by reduced mitochondrial mass (abundance) associated with decreased levels of mitochondrial protein expression and MitoTracker Green labeling (35). Impaired mitochondrial function could render neurons more vulnerable to apoptosis mediated by oxidative stress and free-radical injury. Apart from aging, the underlying mechanisms of impaired mitochondrial function and reduced mitochondrial mass in AD are not known. However, the findings herein suggest that over-expression of AD7c-NTP may contribute to this mechanism of neuronal cell death. In this regard, it is noteworthy that the uninduced Lac F-AD7c cells manifested reduced levels of MTT activity,

although the cells remained viable and AD7c-NTP expression was at a very low level. This suggests that even very low levels of AD7c-NTP over-expression may compromise mitochondrial function, and thereby predispose neuronal cells to oxidative stress and free radical injury.

In cells that were induced to over-express the *AD7c-NTP* gene, apoptosis was mediated by increased expression of the pro-apoptosis gene products, p53 and CD95. In AD, as well as in other neurodegenerative diseases, p53- and CD95-mediated apoptosis are important mechanisms of cell loss (3, 10). The levels of both p53 and CD95 increased with AD7c-NTP expression, and were not increased in cells that were induced to express the *CAT* gene, and recent studies further demonstrated that neuronal cells induced with IPTG to express a cDNA encoding β-galactosidase or luciferase also remained viable (data not shown). Results from previous studies suggest that the accumulation of amyloid-β in brains with AD may be an important mediator of apoptosis because increased levels of Bax and p53 immunoreactivity have been localized within and around amyloid-β deposits in senile plaques (3, 4, 58, 59). Experimentally, amyloid-β has been shown to be neurotoxic or to induce pro-apoptosis and inhibit cell survival gene expression (13, 15–18, 60–62) and activate oxidative stress-related genes (17, 63, 64). Moreover, amyloid-β-induced cellular degeneration can be rescued or prevented by treatment with antioxidant or free radical scavenger agents (61). Therefore, amyloid-β deposits in the brain may indirectly contribute to cell loss in AD due to activation of pro-apoptosis genes.

We investigated the potential role of amyloid-β-accumulation in AD7c-NTP-induced neuronal cell death by measuring amyloid-β immunoreactivity directly in the cultured cells stimulated with different concentrations of IPTG. Those studies demonstrated that AD7c-NTP over-expression was not associated with increased levels of amyloid-β immunoreactivity, thus making it is unlikely that the AD7c-NTP-induced apoptosis and pro-apoptosis gene activation were mediated indirectly through amyloid-β cytotoxicity. This finding is pertinent because the in vivo investigations of human brain tissue also demonstrated a lack of correlation between AD7c-NTP over-expression and amyloid-β immunoreactivity in AD (39). In this regard, it is noteworthy that only some amyloid-β deposits are surrounded by p53- or Bax-immunoreactive fibrils (3, 58, 59), suggesting that other factors may be required to link amyloid-β to neurodegeneration.

The mechanisms by which AD7c-NTP over-expression leads to increased apoptosis, impaired mitochondrial function, and increased levels of pro-apoptosis gene expression in neuronal cells are not known. One possible interpretation of the data is that that high-level expression of a membrane-associated protein such as AD7c-NTP could overwhelm the endoplasmic reticulum and thereby activate cell death pathways. However, this is an unlikely scenario because over-expression aspartyl-asparaginyl β-hydroxylase, which is also a transmembrane protein, does not cause apoptosis in PNET2 neuronal cells. Alternatively, the association between AD7c-NTP immunoreactivity and dementia-associated cytoskeletal lesions in AD brains may involve indirect mechanisms leading to the co-accumulation of hyperphosphorylated cytoskeletal proteins such as phospho-tau. Although the studies herein demonstrated increased levels of phospho-tau immunoreactivity with AD7c-NTP over-expression, it is not certain if this phenomenon is caused by other factors related to the activation of pro-apoptosis pathways. For example, in recent studies we demonstrated that oxidative stress and free radical injury induce some Alzheimer-type molecular abnormalities including p53-mediated apoptosis, impaired mitochondrial function and accumulation of phospho-tau in neuronal cells (36, 37). Therefore, a potential mechanism by which AD7c-NTP over-expression leads to similar neuronal abnormalities and contributes to the AD neurodegeneration cascade is to promote intracellular oxidative injury. Future experiments will be designed to test this hypothesis using in vivo and in vitro models.

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Received September 14, 2000
Revision received November 10, 2000
Accepted November 13, 2000